National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

University of Alberta

The Role of Capsaicin-Sensitive Pulmonary Afferent Nerves in Guinea-pigs

By

Abbas Jafarian ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

in

Pharmaceutical Sciences
(Pharmacology)

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Spring 1996

National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-10599-7

Canada

University of Alberta
Library Release Form

Name of Author: Abbas Jafarian

Title of Thesis: The Role of Capsaicin-Sensitive Pulmonary Afferent Nerves in Guinea-pigs

Degree: Doctor of Philosophy.

Year this Degree Granted: 1996

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material from whatever without the author's prior written permission.

A. Ja

Abbas Jafarian
No. 1, 56 Alley
Yasar St. (Ostandari St.)
Shahrekord, 88157
Iran

January 31, 1996

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Role of Capsaicin-Sensitive Pulmonary Afferent Nerves in Guinea-pigs" here submitted by Abbas Jafarian in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmacology).

Dr. D.F. Biggs, Supervisor

Dr. M.R. Suresh, Supervisory Committee Member

Dr. S.F.P. Man, Supervisory Committee Member

Dr. R.L. Jones, Committee Member

Dr. L.I. Wiebe, Committee Member

Dr. I.W. Rodger, External Examiner

Dr. C.G. Irvin, External Reader

January 29, 1996

اطلب العلم من المهد الی اللحد

ز گهواره تا گور دانش بجوی

Search for knowledge from birth to death

Prophet Muhammad (PBUH)

to my best friend and beloved wife, whose encouragement were my best support
during theses years

to my parents for their best efforts to raise me

to my lovely daughters

ABSTRACT

We studied the role of sensory C-fibres in inducing bronchoconstriction in guinea-pigs by using a monoclonal anti-substance P antibody (α-SP MAb), selective neurokinin receptor (NKR) agonists and antagonists. The relative affinities of the α-SP MAb for SP, neurokinin A (NKA), SP fragments and NKR antagonists were estimated by an inhibition ELISA. Our findings confirmed that the α-SP MAb binds to epitopic sites on SP's C-terminal. Amino acids 6 and 7, and, to a lesser extent, 8 and 9, determine affinity.

We characterized bronchoconstrictor responses to SP and its fragments, NKA, selective NKR agonists and capsaicin (all iv) using selective NK1 and NK2 receptor antagonists in guinea-pigs. C-terminal fragments of SP induced dose-dependent increases in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L); their rank order of potency was: $SP^{4-11} >> SP^{5-11} = SP^{3-11} = SP^{2-11} > SP = SP^{6-11}$. The selective NK1 receptor antagonists shifted the dose-response curves to SP, SP fragments and selective NKR agonists to the right. The selective NK2 receptor antagonist significantly reduced increases in R_L and E_L induced by SP^{4-11}, NKA and capsaicin. Combinations of NK1 and NK2 receptor antagonists shifted dose-response curves to SP, SP fragments, NKA and capsaicin to the right more than NK1 or NK2 receptor antagonists alone. These findings show that SP, SP^{5-11}, SP^{3-11}, SP^{2-11} and SP^{6-11} induce bronchoconstriction mainly via NK1 receptors; SP^{4-11} acts via NK1 and NK2 receptors. Also, these findings indicated that capsaicin-induced bronchospasm is mediated mainly via NK2 receptors.

Whether passive immunization with α-SP MAb prevented bronchospastic responses to SP, NKA and capsaicin, *in vivo*, was determined in groups of guinea-pigs by recording R_L and E_L. α-SP MAb, given iv, markedly inhibited increases in R_L and E_L

induced by SP and NKA, but not by capsaicin. However, when given ip and iv, it greatly reduced pulmonary responses to capsaicin. These findings indicate that passive immunization with α-SP MAb can prevent the bronchospastic effects of exogenous SP and NKA or endogenously released tachykinins (TK), SP and NKA, in guinea-pigs.

Pretreatment with atropine and vagotomy did not change bronchospastic responses to capsaicin. By contrast, the ganglionic blocker mecamylamine and the non-specific β-adrenoceptor blocker nadolol increased responses to capsaicin. The effects of combinations of mecamylamine and nadolol were additive. We concluded that the endogenous release of the TK, SP and NKA, mediated bronchoconstriction induced by capsaicin and there were no central or local cholinergic reflexes involved in these responses.

I am grateful to:

Dr. D.F. Biggs for his invaluable guidance and encouragement during the course of this work,

Dr. M.R. Suresh and Dr. S.F.P. Man, members of my supervisory committee for their encouragement and suggestions at various stages during the research,

Dr. R.L. Jones and Dr. L.I. Wiebe for their help during this research,

Ministry of Health and Medical Education, Islamic Republic of Iran, for its financial support through a Ph.D. scholarship,

And all of my teachers during these years.

Table of contents:

CHAPTER 1
INTRODUCTION

INTRODUCTION . . . 1

1.1. Regulation of bronchomotor tone . . . 2
  1.1.1. Afferent innervation . . . 5
  1.1.2. Cholinergic innervation . . . 7
    1.1.2.1. Modulation of efferent parasympathetics . . . 8
    1.1.2.2. Muscarinic receptor subtypes in airways . . . 10
    1.1.2.3. Airway diseases and the cholinergic system . . . 11
  1.1.3. Sympathetic innervation . . . 11
    1.1.3.1. β-adrenoceptors . . . 13
    1.1.3.2. α-adrenoceptors . . . 15
  1.1.4. NANC system . . . 18
    1.1.4.1. Inhibitory NANC mechanisms . . . 18
    1.1.4.2. Excitatory NANC mechanisms . . . 25
  1.1.5. Submucosal regulation . . . 25
    1.1.5.1. Myoneural junction . . . 26
  1.1.6. Epithelial modulation . . . 26

1.2. Capsaicin and primary afferent neurons . . . 27
  1.2.1. The capsaicin receptor . . . 28
  1.2.2. Capsaicin-induced desensitization . . . 30
  1.2.3. Afferent-efferent dual function of capsaicin-sensitive sensory neurons . . . 31
  1.2.4. Heterogeneity of capsaicin-sensitive sensory neurons . . . 34
  1.2.5. Coexistence of neuropeptides in capsaicin-sensitive neurons . 35

1.3. NANC excitatory system . . . 36
  1.3.1. Tachykinins . . . 36
    1.3.1.1. TK and their expression . . . 37
    1.3.1.2. TK receptors . . . 37
      1.3.1.2.1. Structural characterization of the NKR . . . 40
      1.3.1.2.2. Distribution of NKR . . . 40
    1.3.1.3. Enzymatic degradation of TK in the airways . . . 42
    1.3.1.4. Mechanisms of action of TK . . . 43
    1.3.1.5. TK and airway pathophysiology . . . 44
    1.3.1.6. TK and pulmonary circulation . . . 48
    1.3.1.7. TK and mucus secretion . . . 48
    1.3.1.8. TK and cells of inflammation . . . 48
    1.3.1.9. TK and airway responses to antigen . . . 49
    1.3.1.10. NKR antagonists . . . 50
  1.3.2. CGRP . . . 52
    1.3.2.1. Origin and secretion of CGRP . . . 53
    1.3.2.2. Metabolism of CGRP . . . 54

1.3.2.3. CGRP receptors 54
1.3.2.4. Biologic effects of CGRP 56
1.4. Methods of assessment of airway's responsiveness 58
1.4.1. Direct measurement of smooth muscle function 58
1.4.2. Indirect measurement of smooth muscle function 58

1.5. Antibodies as tools for studying mechanisms of action of drugs or endogenous mediators 61
1.5.1. Monoclonal antibodies (MAb) 62
1.5.2. Production of MAb 63
1.5.3. Purification of MAb 65

OBJECTIVES 69

CHAPTER 2
DIFFERENTIATION OF THE EFFECTS OF BRONCHOSPASTIC AGONISTS ON THE CONDUCTING AND DISTENSIBLE AIRWAYS OF ANESTHETIZED, PARALYSED GUINEA-PIGS USING COMPUTERIZED MEASUREMENTS OF PULMONARY FLOW RESISTANCE (R_L) AND DYNAMIC PULMONARY ELASTANCE (E_L) 70

2.1. INTRODUCTION 71

2.2. METHODS AND MATERIALS 72
2.2.1. Animals 72
2.2.2. Methods 73
2.2.2.1. Measurements 73
2.2.2.2. Evaluation of system for measuring of R_L and E_L . . 76
2.2.2.3. Nerve stimulation 76
2.2.2.4. Agonists given iv 77
2.2.3. Materials 77
2.2.4. Statistical analyses 77

2.3. RESULTS 78
2.3.1. Measurements of resistance and elastance 78
2.3.2. Effects of vagal stimulation 82
2.3.3. Responses to histamine 88
2.3.4. Responses to 5HT 88
2.3.5. Responses to SP 88
2.3.6. Responses to NKA 95

2.4. DISCUSSION 95

CHAPTER 3
PASSIVE IMMUNIZATION WITH AN ANTI-SUBSTANCE P ANTIBODY PREVENTS SUBSTANCE P- AND NEUROKININ A-INDUCED BRONCHOSPASM IN ANESTHETIZED GUINEA-PIGS 100

3.1. INTRODUCTION 101

3.2. METHODS AND MATERIALS 101
- 3.2.1. Methods 101
  - 3.2.1.1. Production of α-SP MAb 101
    - 3.2.1.1.1 Classical method 102
    - 3.2.1.1.2. Bioreactor (artificial capillary module) . . 102
  - 3.2.1.2. Purification of α-SP MAb 103
  - 3.2.1.3. Preparation of SP-BSA conjugate 105
  - 3.2.1.4. α-SP MAb ELISA 105
  - 3.2.1.5. Measurement of airways' responsiveness 107
- 3.2.2. Materials 108
- 3.2.3. Statistical analyses 108

3.3. RESULTS 109
- 3.3.1. Production and purification of α-SP MAb 109
- 3.3.2. Activity and specificity of α-SP MAb 111
- 3.3.3. Effect of α-SP MAb on bronchospasm induced by SP, NKA, or methacholine 114

3.4. DISCUSSION 116

CHAPTER 4
CHARACTERIZATION OF THE MONOCLONAL ANTI- SUBSTANCE P ANTIBODY DERIVED FROM NC1/34 123

4.1. INTRODUCTION 124

4.2. METHODS AND MATERIALS 125
- 4.2.1. Methods 125
  - 4.2.1.1. Preparation of α-SP MAb 125
  - 4.2.1.2. Preparation of SP-BSA conjugate 125
  - 4.2.1.3. Measurement of relative affinities by ELISA 125
  - 4.2.1.4. Synthesis of peptides 126
  - 4.2.1.5. Direct ELISA on SPOTs membrane (dot immunoassay) 129
- 4.2.2. Materials 130
- 4.2.3. Statistical analyses 130

4.3. RESULTS 131
    4.3.1. Relative affinities of α-SP MAb for SP, its fragments and selective NKR antagonists 131
    4.3.2. Alanine scanning 132

4.4. DISCUSSION 140

CHAPTER 5
PHARMACOLOGIC CHARACTERIZATION OF BRONCHOSPASM INDUCED BY SUBSTANCE P AND SUBSTANCE P FRAGMENTS IN GUINEA-PIGS 144

5.1. INTRODUCTION 145

5.2. METHODS AND MATERIALS 146
    5.2.1. Animals 146
    5.2.2. Methods 147
        5.2.2.1. Measurement of airways' responsiveness 147
    5.2.3. Materials 148
    5.2.4. Statistical analyses 148

5.3. RESULTS 148
    5.3.1. Bronchopulmonary responses to SP and SP fragments 148
    5.3.2. Effects of NKR antagonists on bronchopulmonary responses induced by SP and SP fragments 149

5.4. DISCUSSION 159

CHAPTER 6
MECHANISMS INVOLVED IN BRONCHOPULMONARY RESPONSES TO CAPSAICIN 164

6.1. INTRODUCTION 165

6.2. METHODS AND MATERIALS 166
    6.2.1. Animals 166
    6.2.2. Methods 167
        6.2.2.1. Pulmonary measurements 167
    6.2.3. Materials 168
    6.2.4. Statistical analyses 169

6.3. RESULTS 169
    6.3.1. Bronchopulmonary responses to capsaicin and effects of autonomic blockers 169

6.3.2. Effects of α-SP MAb on bronchopulmonary responses to capsaicin 170

6.3.3. Bronchopulmonary responses induced by SP, NKA, [Sar^9,Met(O$_2$)^{11}]-SP, [β-Ala^8]-NKA and capsaicin 174
6.3.4. Effects of NKR antagonists on bronchopulmonary responses induced by SP, NKA, [Sar^9,Met(O$_2$)^{11}]-SP, [β-Ala^8]-NKA and capsaicin 174
6.3.5. Effects of hCGRP and hCGRP$^{8-37}$ on bronchopulmonary responses to SP, NKA or capsaicin 175

6.4. DISCUSSION 185

CHAPTER 7
EFFECTS OF RS-ALBUTEROL ON THE DEVELOPMENT OF ANTIGEN-MEDIATED AIRWAY HYPERREACTIVITY IN GUINEA-PIGS 190

7.1. INTRODUCTION 191

7.2. METHODS AND MATERIALS 191
7.2.1. Methods 191
7.2.1.1. Preparation of α-OA Ab containing serum 191
7.2.1.2. Passive immunization of guinea-pigs 193
7.2.1.3. Aerosol exposure 193
7.2.1.4. Measurement of airways' responsiveness 193
7.2.1.5. Preparation of osmotic pumps 194
7.2.1.6. Implantation of pumps 194
7.2.1.7. Removal of pumps 195
7.2.1.8. Evaluation of pump function 195
7.2.1.9. Bronchial alveolar lavage 195
7.2.1.10. Protocol 195
7.2.2. Animals 195
7.2.3. Materials 196
7.2.4. Statistical analyses 198

7.3. RESULTS 198
7.3.1. Implantation of pumps 198
7.3.2. Evaluation of pump function 198
7.3.3. Effects noted upon aerosol exposure 199
7.3.4. Baseline measurements of R_L and E_L 199
7.3.5. Dose-response curves to histamine and LTC_4 201
7.3.6. Differential cell counts 206

7.4. DISCUSSION 208

CONCLUSIONS AND SUMMARY 211

REFERENCES 218

List of tables:

TABLE 1.1 Classification of afferent innervation 3

TABLE 1.2 Mediators affecting airway smooth muscle 20

TABLE 4.1 Amino acid sequences of SP^{6-11} and SP analogues synthesized by SPOTs analysis 128

TABLE 4.2 Relative affinities of α-SP MAb for SP and SP fragments 132

TABLE 5.1 The relative bronchoconstrictor activities of substance P and SP fragments 150

TABLE 5.2 Antagonism of the bronchospastic effects of substance P and SP fragments by selective neurokinin antagonists in anesthetized guinea-pigs 160

TABLE 6.1 Time (s) required to achieve maximal bronchospasm (peak response) after injection of agonists iv (t max) and the half life ($t_{1/2}$) of the peak response 175

TABLE 7.1 Experimental design for studying the effects of RS-albuterol 196

TABLE 7.2 Volumes (mL) delivered (2.00 mL - volume aspirated) by each osmotic pump 199

List of figures:

Fig 1.1 Schematic representation of sympathetic and parasympathetic innervation to airway smooth muscle of guinea-pigs 9

Fig 1.2 Schematic representation of non-adrenergic non-cholinergic (NANC) excitatory and inhibitory and parasympathetic innervation to airway smooth muscle of guinea-pigs 17

Fig. 1.3 Schematic representation of innervation of guinea-pig airway smooth muscle 24

Fig. 1.4 Structure of the NK1 receptor 39

Fig. 1.5 Regulation of cellular Ca^{2+} by PLC-linked NK1 receptor in exocrine gland cells 45

Fig. 1.6 Chemical structure of non-peptide neurokinin receptor antagonists CP 96,345, CP 99,994, RP 67,580 and SR 48,968 51

Fig. 1.7 Schematic diagram of an artificial capillary system 64

Fig. 2.1 Schematic representation of the system for measuring pulmonary flow resistance and dynamic pulmonary elastance 75

Fig. 2.2 The relationship between pump speed or length of PE240 tube and resistance 79

Fig. 2.3 The relationship between pump speed and pulmonary flow resistance in anesthetized guinea-pigs 80

Fig. 2.4 The relationship between pump speed and dynamic pulmonary elastance in anesthetized guinea-pigs 81

Fig. 2.5 Changes in pulmonary flow resistance and dynamic pulmonary elastance to varying numbers of stimuli applied to the vagi in guinea-pigs 83

Fig. 2.6 Effects of autonomic blockers on pulmonary flow resistance in response to varying stimuli applied to the vagi in guinea-pigs 84

Fig. 2.7 The relationship between percent change in pulmonary flow resistance or dynamic pulmonary elastance and the numbers of stimuli applied to the vagi in guinea-pigs 85

Fig. 2.8 Changes in pulmonary flow resistance and dynamic pulmonary elastance to various doses of methacholine guinea-pigs 86

Fig. 2.9 The relationship between percent change in pulmonary flow resistance or dynamic pulmonary elastance and dose of methacholine in guinea-pigs 87

Fig. 2.10 Changes in pulmonary flow resistance and dynamic pulmonary elastance to various doses of histamine in guinea-pigs 89

Fig. 2.11 The relationship between percent change in pulmonary flow resistance or dynamic pulmonary elastance and dose of histamine in guinea-pigs 90

Fig. 2.12 Changes in pulmonary flow resistance and dynamic pulmonary elastance to various doses of serotonin in guinea-pigs 91

Fig. 2.13 The relationship between percent change in pulmonary flow resistance or dynamic pulmonary elastance and dose of serotonin in guinea-pigs 92

Fig. 2.14 Changes in pulmonary flow resistance and dynamic pulmonary elastance to various doses of substance P in guinea-pigs 93

Fig. 2.15 The relationship between percent change in pulmonary flow resistance or dynamic pulmonary elastance and dose of substance P guinea-pigs 94

Fig. 2.16 Changes in pulmonary flow resistance and dynamic pulmonary elastance to various doses of neurokinin A in guinea-pigs 96

Fig. 2.17 The relationship between percent change in pulmonary flow resistance or dynamic pulmonary elastance and dose of neurokinin A in guinea-pigs 97

Fig. 3.1 Protein A-sepharose chromatography and thiophilic affinity chromatography of supernatant from NC1/34 heterohybridoma 110

Fig. 3.2 Dilution curve for the anti-substance P antibody (α-SP MAb) 112

Fig. 3.3 Relative affinities of anti substance P antibody for SP, NKA or CGRP 113

Fig. 3.4 Relationship between α-SP MAb dilution and maximal OD 115

Fig. 3.5 The effect of anti-substance P antibody (α-SP MAb) or α-HRPO MAb on the changes in pulmonary flow resistance in response to various doses of SP, neurokinin A, or methacholine 117

Fig. 3.6 The effect of anti-substance P antibody (α-SP MAb) or α-HRPO MAb on the changes in dynamic pulmonary elastance in response to various doses of SP, neurokinin A, or methacholine 118

Fig. 3.7 The effect of anti-substance P antibody (α-SP MAb) on changes in pulmonary flow resistance in response to various doses of SP 119

Fig. 4.1 Dilution curve for the anti-substance P antibody 133

Fig. 4.2 Relative affinities of anti-substance P antibody for SP or N-terminal fragments of SP 134

Fig. 4.3 Relative affinities of anti-substance P antibody for SP or SP$^{7-8}$, SP$^{8-9}$, SP$^{9-10}$, SP$^{10-11}$ and SP^{11} 135

Fig. 4.4 Relative affinities of anti-substance P antibody for SP or selective tachykinin receptor antagonists 136

Fig. 4.5 Relative affinities of anti-substance P antibody for SP or SP$^{6-11}$ and SP$^{7-11}$ 137

Fig. 4.6 Relative affinities of anti-substance P antibody for SP or SP$^{2-11}$, SP$^{3-11}$, SP$^{4-11}$ and SP$^{5-11}$ 138

Fig. 4.7 Relative affinities of anti-substance P antibody for SP or SP$^{8-11}$ and SP$^{9-11}$ 139

Fig. 5.1 Changes in pulmonary flow resistance in response to various doses of SP and SP fragments in guinea-pigs 151

Fig. 5.2 Changes in dynamic pulmonary elastance in response to various doses of SP and SP fragments in guinea-pigs 152

Fig. 5.3 The effects of iv administration of selective tachykinin receptor antagonists on the changes in pulmonary flow resistance induced by various doses of SP 153

Fig. 5.4 The effects of iv administration of selective tachykinin receptor antagonists on the changes in pulmonary flow resistance induced by various doses of SP$^{2-11}$ 154

Fig. 5.5 The effects of iv administration of selective tachykinin receptor antagonists on the changes in pulmonary flow resistance induced by various doses of SP^{3-11} 155

Fig. 5.6 The effects of iv administration of selective tachykinin receptor antagonists on the changes in pulmonary flow resistance induced by various doses of SP^{4-11} 156

Fig. 5.7 The effects of iv administration of selective tachykinin receptor antagonists on the changes in pulmonary flow resistance induced by various doses of SP^{5-11} 157

Fig. 5.8 The effects of iv administration of selective tachykinin receptor antagonists on the changes in pulmonary flow resistance induced by various doses of SP^{6-11} 158

Fig. 6.1 The effects of autonomic blockers on changes in pulmonary flow resistance in response to various doses of capsaicin 171

Fig. 6.2 The effects of autonomic blockers on changes in pulmonary flow resistance in response to various doses of capsaicin 172

Fig. 6.3 The effects of α-SP MAb on changes in pulmonary flow resistance in response to various doses of capsaicin 173

Fig. 6.4 The effects of selective tachykinin receptor antagonists on changes in pulmonary flow resistance in response to various doses of NKA and SP 176

Fig. 6.5 The effects of selective tachykinin receptor antagonists on changes in dynamic pulmonary elastance in response to various doses of NKA and SP 177

Fig. 6.6 The effects of selective tachykinin receptor antagonists on changes in pulmonary flowresistance in response to various doses of selective NKR agonists 178

Fig. 6.7 The effects of selective tachykinin receptor antagonists on changes in pulmonary flow resistance in response to various doses of capsaicin 179

Fig. 6.8 The effects of selective tachykinin receptor antagonists on changes in pulmonary flow resistance in response to various doses of capsaicin 180

Fig. 6.9 The effects of CGRP on changes in pulmonary flow resistance in response to various doses of SP and NKA 181

Fig. 6.10 The effects of CGRP on changes in pulmonary flow resistance in response to various doses of capsaicin 182

Fig. 6.11 The effects of $CGRP^{8-37}$ on the depressor effects of CGRP 183

Fig. 6.12 The effects of $CGRP^{8-37}$ on changes in pulmonary flow resistance in response to various doses of capsaicin 184

Fig 7.1 Schematic diagram illustrating the protocol for studying the effectes of 197

Fig. 7.2 Baseline values of pulmonary flow resistance and dynamic pulmonary elastance 200

Fig. 7.3 Changes in pulmonary flow resistance in guinea-pigs following injection of histamine 202

Fig. 7.4 Changes in dynamic pulmonary elastance in guinea-pigs following injection of histamine 203

Fig. 7.5 Changes in pulmonary flow resistance in guinea-pigs following injection of LTC_4 204

Fig. 7.6 Changes in dynamic pulmonary elastance in guinea-pigs following injection of LTC_4 205

Fig. 7.7 Summary of data obtained from differential cell counts 207

List of abbreviations:

5HT	Serotonin
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
Ad	Adrenaline
ALB	Albuterol
ANOVA	Analysis of variance
α-SP MAb	Anti-substance P monoclonal antibody
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DAG	Diacylglycerol
ECS	Extracapillary space
EDRF	Endothelium-derived relaxant factor
E_L	Dynamic pulmonary elastance
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
HRPO	Horse radish peroxidase
IP3	Inositol-1,4,5-trisphosphate
ip	Intraperitoneal
iv	Intravenous
LPS	Large pore size
LTC_4	leukotriene C_4
MAb	Monoclonal antibody
Na^+	Sodium ion
NA	Noradrenaline
NANC	Non-adrenergic non-cholinergic
NEP	Neutral endopeptidase
NKA	Neurokinin A
NKB	Neurokinin B
NKR	Neurokinin receptor(s)
NO	Nitric oxide
NPY	Neuropeptide tyrosine
NS	Normal saline
OA	Ovalbumin
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E_2
PHI	Peptide histidine isoleucine
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
R_L	Pulmonary flow resistance
SDS-PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
SP	Substance P
SPS	Small pore size
T-Gel™	Thiophilic gel
TK	Tachykinin
TKR	Tachykinin receptor
VIP	Vasoactive intestinal peptide

CHAPTER 1

INTRODUCTION

1.1. Regulation of bronchomotor tone

Bronchomotor tone is regulated via the actions and interactions of endogenously secreted mediators or chemicals. Environmental stimuli are converted into electrical impulses that are relayed through the central nervous system (CNS) (Nadel, 1977). These afferent stimuli may themselves be mediators: histamine released during mast cell degranulation activates afferent rapidly adapting receptors (Nadel, 1977). Efferent transmission involves reconversion of electrical impulses into chemical secretion at multiple levels including the CNS, the autonomic ganglia and the synaptic cleft. In the synaptic cleft, mediator secretion (e.g. acetylcholine (ACh), norepinephrine and non-adrenergic non-cholinergic (NANC) mediators) converts the electrical impulses of the efferent autonomic nerves to alter the level of bronchomotor tone (see Table 1.1) (Weiner & Taylor, 1985). This process is initiated by specific binding of mediators to membrane receptors on the airway smooth muscle cells (Weiner & Taylor, 1985).

The neuronal control of airway smooth muscle is complex. The airways contain both afferent and efferent innervation which controls the functions of airway smooth muscle, blood vessels and submucosal glands. In all species, parasympathetic cholinergic nerves are the dominant neural control of airway smooth muscle and stimulation of those leads to bronchoconstriction (Barnes, 1986b). Unlike the parasympathetic innervation, the sympathetic innervation to airway smooth muscle is highly species-specific (Richardson, 1979). It should be noted that even in species which lack a direct sympathetic nerve supply to airway smooth muscle, adrenergic activity has been shown to counteract cholinergic activity (Daniel et al., 1986). Parasympathetic and sympathetic nerves are

TABLE 1.1

Classification of afferent innervation

Afferent innervation	Classification	Function
Slowly adapting receptors (steretch receptors)	Myelinated	Hering-Breuer inflation reflex and reflex bronchodilation
Rapidly adapting receptors (irritant receptors)	Myelinated	bronchoconstriction and cough
C-fibres	Non-myelinated	bronchoconstriction, plasma protein extravasation, mucus secretion, vasodilation and recruitment of inflammatory cells

distributed unevenly throughout the airways. As the diameter of the airways decreases, the density of the innervation decreases (Barnes, 1986b).

Neural responses may be augmented or inhibited at the afferent visceral sensory receptor, at the efferent ganglion, at the central nervous system (CNS), or at the synaptic cleft itself. Simultaneous secretion of multiple mediators at the same postsynaptic site may augment (Poch & Holzman, 1980) or inhibit (Leff & Munoz, 1980) the physiologic effects of each mediator. This may be caused by postjunctional mediator-mediator interactions (Leff & Munoz, 1981) or by secretion of chemotactic factors that attract cells that also secrete mediators that activate smooth muscle contraction. Cells that secrete mediators (e.g., mast cells) are also regulated by a complex series of intracellular events that parallel the membrane transduction and intracellular calcium regulating mechanisms that affect airway smooth muscle. Epithelial cells may also modulate stimuli to airway smooth muscle from the mucosal side via secretion of stimulatory (Cooper et al., 1986) or inhibitory substances (Barnes et al., 1985) directly onto airway smooth muscle. Finally, airway smooth muscle responses must be considered in the context of other factors that can affect airway calibre e.g. edema formation through the process of inflammation and mucus secretion (James et al., 1986). Both of these processes are fundamental components of human asthma. All these actions and interactions between autonomic nervous system and cellular-derived mediators, create complex mechanisms in the regulation of bronchomotor tone.

The autonomic nervous system controls many aspects of airway function and plays an important role in the regulation of airway calibre in health and disease (Barnes, 1986b). In addition to regulation of airway smooth muscle tone, autonomic nerves may influence the secretion of mucus from submucosal glands, the transport of fluid across the airway epithelium, vascular permeability, blood flow in the bronchial circulation and the release of mediators from mast cells and other inflammatory cells (Barnes, 1991b). As there are significant differences in autonomic innervation among different species, it is difficult to extrapolate findings from experimental animals to humans (Leff, 1988). However, due to difficulties involved in obtaining normal human airways and limitations in studying airway tone and secretions in intact humans, the use of animal models is essential.

The notion that autonomic control may be abnormal in asthma and other airway diseases was first proposed by Alexander and Paddock (1921). They showed that injection of the cholinergic agonist pilocarpine caused wheezing in asthmatics but not in normal subjects. Also, they noted that the pilocarpine-induced wheezing was relieved by an injection of epinephrine. Several different autonomic abnormalities have been proposed. These include enhanced cholinergic, α-adrenergic or NANC excitatory mechanisms, or reduced β-adrenergic or NANC inhibitory mechanisms (Nadel & Barnes, 1984).

In the next section I will focus on three major ways by which airways are influenced: 1) from the serosal side of the airway (i.e., autonomic secretion), 2) from the epithelial side (inhibitory effects and excitatory effects) and 3) via modulation of airway smooth muscle contractility. Special emphasis will be placed on autonomic influences.

1.1.1. Afferent innervation

Three different types of afferent fibres have been identified: 1) slowly adapting (stretch) receptors, 2) rapidly adapting (irritant) receptors and 3) C-fibre endings (see Table 1.1) (Barnes, 1986b).

Slowly adapting (stretch) receptors, which are classified as myelinated nerves, are localized mainly to smooth muscle in conducting airways. They mediate the Hering-Breuer inflation reflex, which inhibits sustained inspiratory activity and prolongs expiration at high lung volumes. These receptors mediate reflex bronchodilation by inhibiting vagal tone (Barnes, 1991b). In normal subjects, deep inspiration inhibits efferent parasympathetic output via these afferent receptors (Nadel & Tierney, 1961).

Rapidly adapting receptors, which are also myelinated nerves, are activated by mechanical stimuli, viral infections, nonspecific irritants and a variety of specific mediators including serotonin (5HT), prostaglandin $F_{2\alpha}$ and histamine (Empey et al., 1976). In contrast to slowly adapting receptors, irritant receptors may cause bronchoconstriction by a reflex increase in vagal efferent activity in asthmatics (Barnes, 1991b).

Rapidly adapting receptors in the upper airways are called cough receptors. There is a close relationship between cough receptors and irritant receptors; bronchoconstriction augments and bronchodilation diminishes the threshold to cough (Chausow & Banner, 1983).

A third series of nonmyelinated nerve endings named C-fibres are also found in the airways, usually within the airway epithelium. C-fibres appear to be involved in a complex series of events that previously may have been attributed to rapidly adapting receptors (Barnes, 1986b). In contrast to irritant receptors, these fibres are stimulated selectively by capsaicin and bradykinin (Coleridge & Coleridge, 1984). C-fibre afferents have been implicated in the release of tachykinins (TK), substance P (SP) and neurokinin A (NKA) (Martling et al., 1988). These mediators appear to cause bronchoconstriction via neurokinin receptors (NKR) (Saria et al., 1985; Foulon et al., 1993). Recently there has been more focus on the role of C-fibres and mediators released from them in the pathogenesis of airway diseases. This will be discussed later.

1.1.2. Cholinergic innervation

The parasympathetic nervous system provides the dominant control of neural bronchoconstrictor mechanism in animals and humans. It plays an important role in the regulation of airway tone (Barnes, 1993). It has been suggested that there is imbalance between the excitatory and inhibitory systems with a bias towards increased cholinergic transmission in airway diseases such as asthma and chronic obstructive airway disease (Barnes, 1992). Cholinergic efferent nerves arise in the vagal nuclei of the brainstem and pass down the vagus nerve to synapse in parasympathetic ganglia located in the airway wall. From these ganglia, relatively short postganglionic fibres innervate target cells, such as smooth muscle cells and mucus glands (Barnes, 1993). The density of cholinergic innervation decreases as the airways get smaller and there is none in the terminal bronchioles and the alveolar walls (Barnes et al., 1983).

Electrical stimulation of the vagus nerves releases ACh which activates muscarinic receptors on airway smooth muscle, submucosal gland cells and the epithelium. This results in narrowing of airways and mucus secretion. The onset of narrowing of airways in response to ACh is rapid and it is readily reversible, suggesting that it is mediated by the contraction of airway smooth muscle rather than bronchial wall edema or luminal obstruction with mucus (Nadel, 1980). The fact that this response is potentiated by cholinesterase inhibitors and blocked by the muscarinic receptor antagonist atropine, provides evidence that the vagal nerves cause bronchoconstriction by the release of ACh from the cholinergic nerve terminals. Once present, the ACh rapidly diffuses to the cholinergic receptors which are relatively close to the nerve terminals. At the synaptic cleft, acetylcholinesterase breaks down ACh. The magnitude of bronchospasm induced by ACh in the airways is consistent with the distribution of the pulmonary cholinergic nerves (i.e., decreasing response to ACh from intermediate size bronchi to the small bronchioles and alveolar ducts) (Nadel, 1980).

1.1.2.1. Modulation of efferent parasympathetics

The functions of cholinergic nerves may be modulated by other neurotransmitters or by inflammatory mediators. It has been shown that isoprenaline and norepinephrine reduced cholinergically-induced contraction of canine and guinea-pig tracheal smooth muscle (Danser et al., 1987; McCaig, 1987; Pendry & McLagan, 1991). By contrast, neither sympathetic stimulation nor applied cathecolamine changed mechanical responses to exogenous ACh (McCaig, 1987; Pendry & McLagan, 1991). These findings suggest that catecholamines inhibit ACh release from postganglionic nerve terminals via effects on prejunctional adrenoceptors. Furthermore, it was shown that these effects are mediated via both β- and α_2-adrenoceptors in guinea-pigs and humans (Grundstorm & Andersson, 1985a; Matran et al., 1989; Pendry & McLagan, 1991) (see Fig. 1.1) (see pages 10-14 for more information). Inflammatory mediators such as prostanoids, histamine and 5HT may also modulate parasympathetic neurotransmision. Prostaglandin E_1 and E_2 inhibit (Inoue & Ito, 1986; Deckers et al., 1989), whereas 5HT and thromboxane A_2 potentiate (Sheller et al., 1982; Chung et al., 1985), cholinergic neurotransmision in the airways.

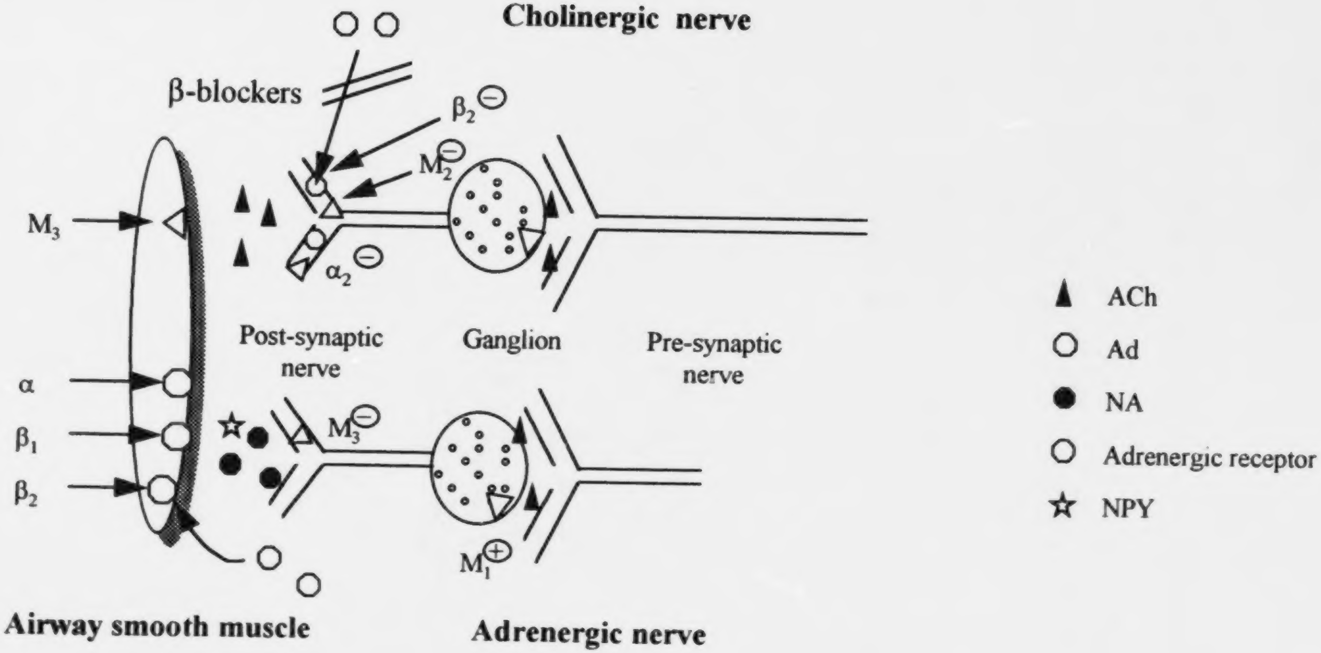

Cholinergic nerve
β-blockers
β_2 ⊖
M_2 ⊖
M_3
α_2 ⊖
Post-synaptic nerve
Ganglion
Pre-synaptic nerve
α
β_1
β_2
M_3 ⊖
M_1 ⊕
Airway smooth muscle
Adrenergic nerve
▲ ACh
○ Ad
● NA
○ Adrenergic receptor
☆ NPY

Fig 1.1

Schematic representation of the sympathetic and parasympathetic innervation to airway smooth muscle in guinea-pigs. Acetylcholine (ACh) released from cholinergic nerves induces contraction via postsynaptic M_3 receptors. The release of ACh is inhibited by its effects on prejunctional M_2 receptors. Circulatory adrenaline (Ad) may exert its bronchodilatory effects on cholinergic neurotransmission via adrenoceptors on airway cholinergic nerves. β-blockers induce bronchoconstriction by inhibiting modulatory effects of circulatory Ad. Activation of α_2-receptors on airway smooth muscle causes bronchoconstriction. α_2-receptors on cholinergic nerves modulate ACh release. M_1 receptors play a facilitatory role in sympathetic ganglia. Stimulation of the sympathetic nerves releases noradrenaline (NA) to β_2-adrenoceptors. ACh may inhibit the release of NA via M_3 receptors on sympathetic nerve endings. + and - indicate facilitatory and inhibitory actions, respectively.

1.1.2.2. Muscarinic receptor subtypes in airways

Five subtypes of muscarinic receptors, M_1 - M_5, have been cloned and from these 3 subtypes have been recognised pharmacologically (Barnes, 1992). Binding studies in guinea-pig whole lung have shown that M_3 receptors are predominant in guinea-pigs, but, in humans, there is relatively equal distribution of M_1 and M_2/M_3 receptors (Mak & Barnes, 1989; Pendry, 1993). M_1 receptors, which are excitatory, are localized to parasympathetic ganglia (Fig. 1.1) (Bloom et al., 1987). M_1 receptors may facilitate ganglion neurotransmission through parasympathetic ganglia (Barnes, 1992) and enhance cholinergic reflex bronchoconstriction in humans (Roffel et al., 1990).

In several species including guinea-pigs and humans, there is evidence for prejunctional M_2 receptors (autoreceptors) on postganglionic airway cholinergic nerves that inhibit the release of ACh (Barnes, 1992). Pilocarpine, which selectively activates M_2 receptors, inhibits cholinergic reflex bronchoconstriction induced by sulphur dioxide in normal subjects, but not in asthmatics (Minette et al., 1989). These findings suggest that there might be dysfunction of autoreceptors in asthma, thus enhancing reflex bronchoconstriction. It has been suggested that M_2 receptors are more susceptible to damage by inflammatory mediators (Barnes, 1992). In guinea-pigs, influenza virus infection resulted in a selective loss of M_2 receptors which could explain airways' hyperresponsiveness after upper respiratory tract viral infection (Fryer & Jacoby, 1991). In some species such as guinea-pigs, sympathetic nerves interact with airway cholinergic nerves. M_2 receptors on sympathetic nerve terminals inhibit the release of norepinephrine from these nerves (Barnes, 1992).

In guinea-pigs, M_3 receptors are localized predominately to the smooth muscle of the proximal airways. In humans, M_3 receptors are abundant in the submucosal glands and airway vascular endothelium (Barnes, 1992). Activation of M_3 receptors causes bronchoconstriction and mucus secretion (Barnes, 1992). M_4 receptors are expressed in rabbit lung, but M_5 receptors have not yet been identified in lung (Barnes, 1992).

1.1.2.3. Airway diseases and the cholinergic system

It has been suggested that there is increased cholinergic activity in airway diseases. This could be as a result of inflammatory mediators, exposure of afferent C-fibre due to damage of epithelium, dysfunction of M_2 receptors, and/or increased in responsiveness of airway smooth muscle to ACh (Barnes, 1991b). However, airways' hyperresponsiveness is not specific for ACh as it is also seen with other bronchoconstrictor agents (Barnes, 1991b). The fact that parasympathetic nerves are the predominant bronchoconstrictor pathway in the airways, the discovery of different subtypes of muscarinic receptors and the modulation of ACh release by prejunctional M_2 receptors suggest important clinical applications for selective anti-muscarinic agents. Thus, selective M_1 and M_3 antagonists, which do not block the modulatory effect of M_2 receptors, may be useful in treatment of airway diseases.

1.1.3. Sympathetic innervation

Adrenergic control of the airways involves the sympathetic nerves (which release norepinephrine), the adrenal medulla (which releases epinephrine) and α- and β-adrenoceptors (Barnes, 1987a). In comparison to the dense pulmonary parasympathetic innervation, sympathetic innervation is generally sparse, but again there is marked variability among species (Richardson, 1979). Sympathetic nerves innervate airway smooth muscle of dog, goat, sheep, cat, pig, calf and guinea-pig, but few, if any, noradrenergic fibres have been demonstrated in airway smooth muscle of rats, rabbits and humans (Pendry, 1993). In guinea-pigs, facilitatory M_1 receptors have been identified on the sympathetic ganglion cell body (Fig. 1.1), but it has not yet been reported in other species (Pendry, 1993).

Sympathetic neurotransmission is an event of brief duration in the airways (Leff & Munoz, 1981b). As for parasympathetic nerves, the ganglionic transmitter is ACh. The postganglionic neurotransmitter, norepinephrine, undergoes rapid uptake or is inactivated by catechol-O-methyltransferase and monoamine oxidase (Pendry, 1993). It has been shown that blockers of neuronal and extraneuronal uptake of noradrenaline potentiate the response to noradrenaline in guinea-pig trachea but, not in guinea-pig bronchi or human airways suggesting the presence of functional adrenoceptors in only guinea-pig trachea (Taylor et al., 1984; Zaagsma et al., 1987).

Studies using electrical field stimulation have shown that tetrodotoxin, but not propranolol, inhibited bronchodilation suggesting the presence of neurally-mediated relaxation. These findings also, suggest that although there is no functional sympathetic innervation of human airway smooth muscle, there is a NANC inhibitory mechanism (Barnes, 1991b).

1.1.3.1. β-adrenoceptors

Despite the fact that sympathetic innervation of airways is sparse, both α- and β-adrenoceptors are present in the airways. The density of β-adrenoceptors increases with decreasing the diameter of airways (Carstairs et al., 1985). β-adrenoceptors are found on many different cell types within the lung, including the smooth muscle of all airways from trachea down to terminal bronchiole but the majority of them are localized to the alveolar walls (Barnes, 1984; Carstairs et al., 1985). β-adrenoceptors induce a potent bronchodilating effect and have the ability to reverse bronchoconstriction regardless of the constrictor stimulus. Activation of β-adrenoceptors causes an increase in concentration of cyclic adenosine monophosphate (cAMP). This leads to activation of cAMP-dependent protein kinase resulting in phosphorylation of proteins involved in airway smooth muscle relaxation (Morley, 1994). This may be mediated via opening potassium channels (Small et al., 1993) or via a direct effect upon G-proteins (Kume et al., 1992). Other effects of β-agonists include inhibition of cholinergic transmission, stimulation of mucus secretion, decreasing the release of mediators from mast cells and, possibly, reduction of plasma extravasation and mucosal edema (Rhoden et al., 1988).

It has been shown that both β_1- and β_2-adrenoceptors are present in the lung. In humans and guinea-pigs, the majority of these receptors are of the β_2 subtype, but this is not so in all species (Pendry, 1993). β-adrenoceptor agonists are potent relaxers of airway smooth muscle *in vitro* and *in vivo* (Barnes, 1991b; Pendry, 1993). These findings and the fact that sympathetic innervation to human airways is sparse (White et al., 1987) suggest that circulating catecholamines may play an important role in regulation of bronchomotor tone (Barnes, 1986c). It has been proposed that the catecholamines, norepinephrine and epinephrine, may inhibit the release of ACh via an action on prejunctional α- or β-adrenoceptors (Fig. 1.1) (Rhoden et al., 1988; Barnes, 1992; Pendry, 1993). These findings together with those of Cerrina et al. (1986) and Goldie et al. (1986) who showed that airways from asthmatic patients failed to relax normally to isoprenaline, suggest that there may be dysfunction of β-adrenoceptors in asthma and other airway diseases (Barnes, 1987a).

It has been shown that β-blockers enhanced the response of several bronchoconstrictor agents in naive animals (Maclagan & Ney, 1979; Ney, 1983; Ballati et al., 1992). Also, similar results were obtained with surgical adrenalectomy (Barnes, 1991b). These findings suggest that circulatory catecholamines may mask existing contractile influences of the parasympathetics (Boushey et al., 1980; Barnes, 1991b). β-adrenergic blockade with propranolol or other β-adrenoceptor blockers causes pronounced bronchoconstriction in asthmatics (Boushey et al., 1980). By contrast, normal persons receiving even greater levels of β-adrenoceptor blockade do not bronchoconstrict spontaneously to propranolol and have virtually identical responses to bronchial challenge compared with pretreatment conditions (McNeill, 1987). Concentrations of circulatory catecholamines are not different in asthmatics at rest and normal subjects (Barnes, 1991b) and, even in severe bronchoconstriction, asthmatics do not demonstrate significant catecholamine secretion (Barnes et al., 1982). Thus, it has been suggested that there may be an impairment of β-adrenoceptors in asthmatics. However, this is disputed as most studies have shown no difference in the function of β-adrenoceptors in untreated asthmatics and normal subjects (Barnes, 1991b).

Recently there has been more interest in the use of β-agonists, as some studies have shown a relationship between the increased morbidity and mortality in asthmatics and use of β-agonists (Mitchell, 1989; Spitzer et al., 1992; Barrett & Storm, 1995). In control and sensitized guinea-pigs, chronic administration of RS-albuterol induced increased airways' responsiveness to several bronchospastic agonists including histamine and leukotriene-C_4 (LTC_4) (Hoshiko & Morley, 1993; Jafarian et al., 1995). Also, in control and sensitized guinea-pigs, chronic administration of rac-fenoterol significantly increased airway responses to ACh both *in vivo* and *in vitro* (Wang et al., 1994). These findings together with increasing awareness of asthma as an inflammatory disease (BTS, 1990; NHLBI, 1991) led to the reconsideration of the use of β_2-agonists in the treatment of asthma and recommendations to use anti-inflammatory agents as first line in asthma therapy.

1.1.3.2. α-adrenoceptors

α-adrenoceptors have been demonstrated in airways of several species but they are less abundant than β-adrenoceptors (Barnes et al., 1979; Barnes, 1992). Like β-adrenoceptors, the density of α-adrenoceptors increases as the diameter of airways decreases (Barnes et al., 1979). Both α_1- and α_2-adrenoceptors are present in the airways (Grundstorm & Andersson, 1985a). The precise mechanism under which α-adrenoceptors regulate bronchomotor tone is not yet understood. It appears that the α-adrenergic response is regulated by a cyclooxygenase products, which are secreted by the muscle itself due to mechanical deformation (Barnes, 1991a). It has been shown that α-adrenoceptor agonists stimulate secretion from serous submucosal glands, induce histamine release from mast cells and induce vasoconstriction (Barnes, 1986b).

The role of α-adrenoceptor stimulation in bronchial hyperreactivity has been studied by assessing the ability of α-adrenergic antagonists to prevent the bronchoconstriction induced by histamine and exercise in patients with asthma (Pendry, 1993). It is proposed that the contractile response to noradrenaline is predominately mediated via α_2-adrenoceptors (Pendry, 1993). Also it has been suggested that mast cell mediators enhance α-adrenoceptor function in airway smooth muscle. However, the demonstration of α-adrenoceptors by autoradiography was not very successful and α-blockers failed to induce bronchodilation (Barnes, 1992). α-antagonists such as phentolamine and thymoxamine inhibited bronchoconstriction induced by histamine and allergen (Patel & Kerr, 1975). However, the poor specificity of these drugs makes interpretation difficult. Other effects of these drugs such as antihistaminic effects or potassium channel activation may account for bronchodilatory responses to them (Barnes, 1991b). Furthermore, prazosin, a selective α_1-antagonist, showed little benefit in asthma, either in challenge studies (Barnes et al., 1981) or chronic control of airway function (Baudouin et al., 1988). Although activation of α_2-adrenoceptors causes bronchoconstriction in guinea-pigs but not in humans, α_2-adrenoceptors inhibit both cholinergic and NANC excitatory mechanisms (Figs. 1.1-1.2) (Grundstorm & Andersson, 1985b). The role of α-adrenergic activity in the regulation of mucus secretion in the airways is another pathway for the involvement of α-adrenergic activity in patients with asthma (Boushey et al., 1980). Also, α-adrenoceptors may indirectly influence airway responsiveness by regulating airway blood flow (Barnes, 1992).

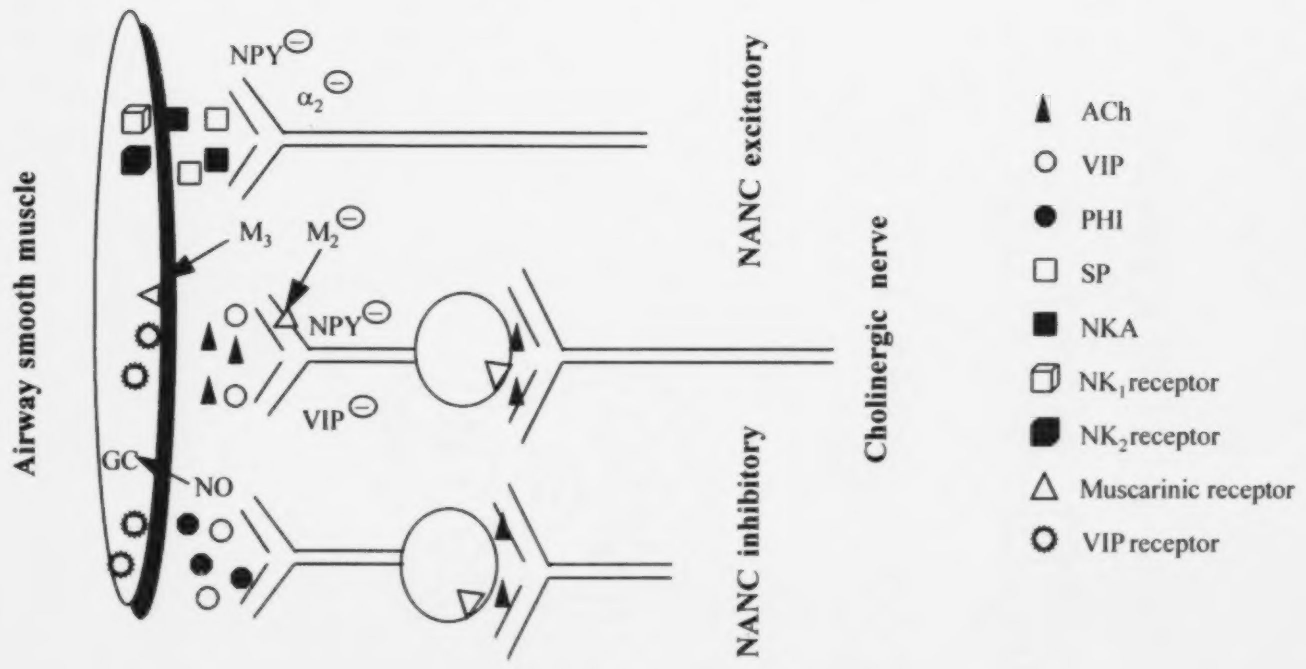

Airway smooth muscle
NPY⊖
α2⊖
M3
M2⊖
NPY⊖
VIP⊖
GC
NO
NANC excitatory
Cholinergic nerve
NANC inhibitory
ACh
VIP
PHI
SP
NKA
NK1 receptor
NK2 receptor
Muscarinic receptor
VIP receptor

Fig 1.2

Schematic representation of non-adrenergic non-cholinergic (NANC) excitatory and inhibitory and parasympathetic innervation to airway smooth muscle of guinea-pigs. Acetylcholine (ACh) released from cholinergic nerves induces contraction via postsynaptic M_3 receptors. The release of ACh is inhibited by its effects on prejunctional M_2 receptors. Tachykinins, substance P (SP) and neurokinin A (NKA), induce bronchoconstriction via NK1 and NK2 receptors. Neuropeptide Y (NPY) modulates release of sensory neuropeptides. Vasoactive intestinal peptide (VIP) and nitric oxide (NO) inhibit cholinergic transmission in guinea-pig airways. VIP and PHI act via binding to their receptors on airway smooth muscle, while NO directly binds to guanylate cyclase. + and - indicate facilitatory and inhibitory actions, respectively.

1.1.4. NANC system

Classically, the autonomic nervous system has been described as consisting of two components; the cholinergic and the adrenergic nerves (Lammers et al., 1992). However, the presence of NANC mechanisms involved in autonomic control of airways has been demonstrated *in vitro* and *in vivo* (Barnes, 1986b; Barnes, 1986d, Pendry, 1993). Both inhibitory and excitatory NANC nerves have been described and studies have shown that neuropeptides, that are localized to classical autonomic nerves, may act as neurotransmitters of these systems (Polak & Bloom, 1986; Barnes, 1988). Despite the large number of studies on NANC responses, our knowledge about the role of these neurally mediated smooth muscle responses in the control of airway smooth muscle tone is lacking. In this section I will focus on the main findings on the role of NANC responses in the control of airway smooth muscle tone.

1.1.4.1. Inhibitory NANC mechanisms

Studies *in vitro* and *in vivo* have shown that one role of NANC inhibitory nerves is to relax airway smooth muscle in several species including humans (Boushey et al., 1980, Michoud et al., 1987). In isolated human airway smooth muscle, orthodromic stimulation of the vagus induced a contractile response that is blocked by pretreatment with atropine and tetrodotoxin suggesting the involvement of parasympathetic nerves. This is followed by a relaxant response which is not affected by β-adrenoceptor blockade but, is affected by tetrodotoxin (Taylor et al., 1984; De Jongste et al., 1987). These findings suggest that neural mechanisms are responsible for this response and that this relaxation is of non-adrenergic origin. However, in guinea-pig tracheal or canine bronchial smooth muscle, the relaxant response to orthodromic stimulation of vagus is partially blocked by β-adrenoceptor blockade (Taylor et al., 1984). Thus, in these species, both adrenergic and NANC mechanisms are involved in relaxation of airway smooth muscle. Studies *in vivo* support the involvement of NANC inhibitory mechanisms in modulation of airway smooth muscle contraction (Irvin et al., 1982; Matsumoto et al., 1985).

As NANC inhibitory nerves are the major bronchodilatory pathway in human airways, it has been suggested that impairment of this system may contribute to the pathogenesis of asthma. Vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), neuropeptide tyrosine (NPY) and nitric oxide (NO) are all possible contributors to NANC inhibitory mechanisms (Barnes, 1986b; Lundberg, 1993). Table 1.2 shows NANC and other mediators affecting airway smooth muscle and their specific effects.

VIP-immunoreactivity is often associated with cholinergic nerves suggesting the coexistence of VIP and ACh (Lundberg et al., 1987). VIP acts via its receptors located on bronchial smooth muscle, airway glands, epithelium and blood vessels (Pendry, 1993). VIP conteracts the bronchoconstrictor activity of ACh and also inhibits its release (Fig. 1.2) (Barnes, 1992). *In vitro*, VIP relaxes airway smooth muscle of several species by activating adenylate cyclase (Lazarus et al., 1986; Rhoden & Barnes, 1990). Also, in humans, inhalation of VIP protects against histamine-induced bronchoconstriction (Barnes & Dixon, 1984). However, inhalation of VIP by itself produced no bronchodilation in human airways (Said, 1982; Barnes & Dixon, 1984). This could be explained by the

TABLE 1.2

Mediators affecting airway smooth muscle

Classification	Mediators	Effects
Parasympathetic	ACh	bronchoconstriction, increased mucus secretion and vasodilation
	VIP, PHI, PHM and NPY	bronchodilation
Sympathetic	noradrenaline and NPY	bronchodilation
NANC inhibitory	VIP, PHI, NPY and NO	bronchodilation
NANC excitatory	SP and NKA	bronchoconstriction, mucus secretion, plasma protein extravasation and recruitment of inflammatory cells
	CGRP	vasodilation, increasing blood flow and modulatory effects on airway smooth muscle?

inability of the relatively large peptide to get to its receptors in the airways, by enzymatic breakdown or by the limited doses used due to the profound cardiovascular effects of VIP. Also, it has been shown that VIP immunoreactivity is reduced in airways of severe asthmatics compared to normal subjects (Pendry, 1993).

Electrical field stimulation of tracheal preparations causes the release of VIP (Matsuzuki et al., 1980; Cameron, 1983). This is abolished by tetrodotoxin suggesting the involvement of the autonomic nervous system (Matsuzuki et al., 1980; Cammeron, 1983).

The amount of VIP released correlates with the magnitude of NANC relaxation (Cammeron, 1983). Also, antibody to VIP and incubation with chymotrypsin, an enzyme responsible for VIP degradation, reduced NANC relaxation (Ellis & Farmer, 1989).

VIP has also been considered as a major candidate for inhibitory NANC vasodilation. However, recent studies have shown that NO synthase (NOS), the enzyme responsible for production of NO from L-arginine, is present in VIP-positive nerves, emphasising the complexity of this issue (Lundberg, 1993).

Also, PHI is probably released with VIP and could be a neurotransmitter of NANC relaxation in airways. PHI is equipotent to VIP as a bronchodilator, but is less potent as a vasodilator suggesting that it might act on different receptors (Palmer et al., 1987b; Yiangou et al., 1987).

NPY is localised in sympathetic neurons and modulates both cholinergic neurotransmission and release of sensory neuropeptides in guinea-pig airways (Fig. 1.2) (Stretton et al., 1989; Pendry, 1993). The content of NPY is much higher in the trachea compared to small bronchi (Martling et al., 1990). Adrenergic blockade does not block the effects of NPY suggesting that NPY-mediated responses are not mediated via adrenoceptors (Stretton & Barnes, 1988).

It has been shown that endothelial relaxant factor is identical to NO (Palmer et al., 1987b). There is increasing evidence that NO may be a putative neurotransmitter in the NANC inhibitory system (Barnes, 1992; Rand, 1992). Although inhaled NO has a larger effect on lung vasculature than on bronchomotor tone, there is evidence suggesting that the NANC inhibitory control of tracheobronchial smooth muscle involves NO as a mediator (Alving et al., 1992; Barnes, 1992; Barnes & Liew, 1995). Thus inhibition of

NOS attenuated the neural bronchodilation response to electrical field stimulation, in guinea-pig and pig trachea. These effects could be partially reversed with L-arginine, but not with D-arginine which is not a substrate for this enzyme (Barnes, 1991a, Tuker et al., 1990; Ellis and Undem, 1992b; Kannan & Johnson, 1992). Furthermore, in human airways, inhibition of NO synthesis by C-N-arginine methyl ester reduced or abolished neural bronchodilation caused by NO (Bai et al., 1992). NO is thought to relax airway smooth muscle via stimulation of guanyl cyclase resulting in production of cyclic 3′, 5′-guanosine monophosphate (Sheng et al., 1991).

Most of the evidence for NO being the inhibitory NANC transmitter for airway smooth muscle relaxation depends on the specificity of NOS inhibitors. There is some evidence that the arginine analogue L-NMMA is not a specific inhibitor of vascular relaxation (Barnes, 1991b) and the endothelium-independent relaxation of blood vessels by VIP is also reduced by L-NMMA (Gaw et al., 1991). Also, studies have shown that VIP-containing nerve fibres can contain NOS, indicating their ability to produce NO (Ceccatelli, et al., 1992; Kummer et al., 1992). Hogman et al. (1993) showed that inhalation of NO caused a mild bronchodilation in asthmatics which suggest a role of NO in NANC inhibitory mechanism. It should be noted that NO may also have harmful effects such as an increase in bronchial blood flow, plasma exudation in the airways and eosinophilia (Kuo et al., 1992; Alving et al., 1993; Barnes & Liew, 1995). These effects are mediated via inducible NOS (Barnes & Liew, 1995) suggesting that specific inhibitors of inducible NOS may be beneficial in asthma and allergic diseases.

Lei et al. (1993) looked at the role of NANC mechanism in the neural bronchoconstriction in guinea-pigs. In this study they investigated whether a neural inhibitory NANC mechanism regulated the magnitude of neural bronchoconstriction *in vivo* in the guinea-pig. They looked at the effects of NOS inhibitors on NANC neural bronchoconstriction induced by electrical vagal stimulation and by capsaicin in the presence of atropine and propranolol. They showed that NOS inhibitors potentiated neural bronchoconstriction induced by vagal stimulation, but not that induced by SP, NKA and capsaicin. They suggested that NO, or NO-releasing material, produced in association with stimulation of vagus nerves, regulated the magnitude of the bronchoconstriction. In this study they used atropine to eliminate the constrictor effect of ACh released from vagal cholinergic nerves and propranolol to prevent adrenergic influences. They suggested that the bronchoconstriction seen in that study was due to SP and NKA released by sensory nerves. Based on their finding that capsaicin-induced bronchoconstriction was not affected by NOS inhibitors, they suggested that cholinergic nerves were the primary source of NO. However, electrical stimulation of vagus nerves in guinea-pigs activates sympathetic efferent, parasympathetic afferent, NANC efferent and parasympathetic efferent nerves (Fig. 1.3). Propranolol and atropine eliminate the postsynaptic effects of sympathetic efferent and parasympathetic efferent nerves, respectively. Thus, the effects of activated NANC efferents and parasympathetic afferents remain intact. Considering this, it is not possible to differentiate the effects of NANC efferent and parasympathetic afferent nerves by just giving atropine and propranolol. It could account for the difference between the effect of NOS inhibitors in vagally stimulated bronchoconstriction and SP, NKA, or

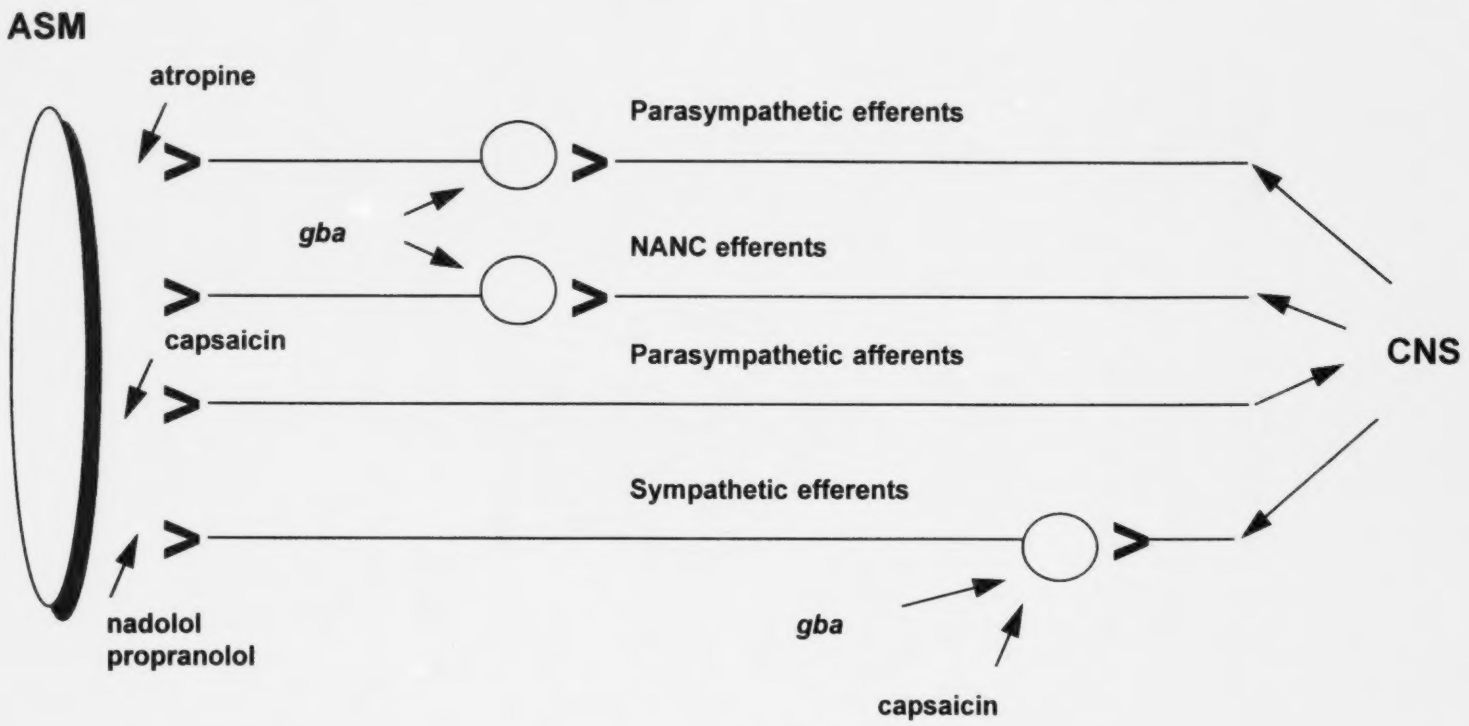

ASM
atropine
Parasympathetic efferents
gba
NANC efferents
capsaicin
Parasympathetic afferents
CNS
Sympathetic efferents
nadolol
propranolol
gba
capsaicin

Fig 1.3

Schematic representation of innervation of guinea-pig airway smooth muscle (ASM). *gba*: ganglionic blocking agent, VS: vagal stimulation capsaicin induced bronchoconstriction in this study. To overcome this problem ganglionic blocking agents could be used. Ganglionic blocking agents block the effects of all sympathetic efferents, parasympathetic efferents and NANC efferents, but not parasympathetic afferents.

1.1.4.2. Excitatory NANC mechanisms

Electrical stimulation of guinea-pig bronchi, and occasionally trachea, produces a component of bronchoconstriction which is resistant to atropine. There is convincing evidence that tachykinins (TK), SP and NKA are neurotransmitters of this response (Andersson & Grundstorm, 1983). These mediators can be released from afferent nerves by capsaicin (Saria et al., 1987). CGRP, another mediator of NANC excitatory system, is costored and coreleased with TK (Maggi & Meli, 1988). See page 36-58 for more details about the excitatory NANC system.

1.1.5. Submucosal regulation

Ultimately, bronchomotor tone is regulated at the level of the airway smooth muscle itself. It begins at the myoneural junction and terminates as the chemical coupling or uncoupling of actin and myosin take place. Many investigators have focused upon the membrane-mediated and intracellular events that translate receptor binding at the plasma membrane of the smooth muscle cell to airway contractility and how these events are modified by 1) membrane polyphosphoinositides and 2) the adenylcyclase-cAMP systems. Both systems appear to mediate or modulate airway smooth muscle tone by controlling the availability of calcium ion (Ca^{2+}) to the contractile apparatus of the cell. However, the interaction between these two systems remains undefined, and the data relating to each therefore are considered separately.

1.1.5.1. Myoneural junction

The myoneural junction is a major locus of action in the neural regulation of bronchomotor tone. It is the final common pathway. Here, electrical impulses from nerves are converted to release of chemical mediators (neurotransmitters) into the synaptic cleft. Circulating humoral agents, reach the smooth muscle membrane through the bloodstream. Studies have shown a potential major role for the local endogenous regulation of smooth muscle tone through 1) local secretion of mediators from circulating cells and fixed tissue elements, 2) local release of inhibitory substances from the adjacent epithelium, and 3) endogenous regulation of smooth muscle tone from substances secreted from the muscle itself. Mediators finally elicit their effects on airway smooth muscle through membrane transduction mechanisms that alter the affinity of binding of actin and myosin.

1.1.6. Epithelial modulation

Different stimuli must traverse the epithelium, which acts as a barrier, to initiate a specific response. Both epithelial cells and the underlying muscle are heterogeneous tissues with innervation, receptor density, and cell metabolism that vary in different species and also they vary as a function of airway generation (Shioya et al., 1987). The epithelium is also a site for the synthesis of proinflmmatory mediators that may be involved in the inflammatory component of asthma. By contrast, the epithelium may synthesize other mediators that counteract smooth muscle contraction. Furthermore, it is a site of metabolism for several mediators which are involved in regulating bronchomotor tone (Flavahan et al., 1985). The epithelium contains the afferent sensory fibres that elicit irritant receptor responses (Nadel, 1977). In asthmatics, it has been shown that there are consistent mucosal alterations with destruction of ciliated epithelial cells (Laitinen et al., 1985). This exposes intraepithelial nerves and mast cells to stimuli such as mechanical or chemical irritants, cellular derived mediators and/or antigen.

Airway smooth muscle responses must also be considered in the context of other factors that may affect airway calibre such as edema formation through the process of inflammation (James et al., 1986) and mucous secretion. Both inflammation and mucus secretion are fundamental components of human asthma.

1.2. Capsaicin and primary afferent neurons

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the active principle of hot peppers, has the unique ability to excite and/or inhibit/degenerate a subpopulation of afferent neurons. These neurons give rise largely, but not exclusively, to non-myelinated axons, and thus are often referred to as C-fibres (Marsh et al., 1988; O'Neill, 1991). In many tissues, capsaicin selectively stimulates a subpopulation of sensory parasympathetic and sympathetic afferent nerves to induce the release of neuropeptides (Richardson, 1979; Manzini et al., 1989; Saria et al., 1988). Thus, in perfused guinea-pig lungs, *in vitro*, low concentrations of capsaicin induced the release of SP, NKA and CGRP (Saria et al., 1984;

Lundberg & Saria, 1987; Martling et al., 1988). However, exposure to large doses of capsaicin caused desensitization of sensory nerves (Maggi et al., 1991a).

1.2.1. The capsaicin receptor

On the basis of the structure-activity relationship of capsaicin analogs, Szolcsanyi and Jansco-Gabor (1975) proposed that capsaicin interacts with a specific receptor. Plant principles including capsaicin, resiniferatoxin and piperine have been shown to activate capsaicin-sensitive primary afferents (Maggi et al., 1990a). Mediators of inflammation and transmitters such as bradykinin, arachidonic acid metabolites, 5HT, histamine and ACh, chemicals from the environment such as smoke, allergens, toluene, ether, formalin and high concentrations of potassium have also shown to have similar effects (Maggi et al., 1990a). Using [^{3}H]resiniferatoxin and capsazepine, a structurally related capsaicin antagonist, competitive binding studies indicated the presence of a specific receptor with a single binding site, the so called the "vanilloid" receptor (Szallasi & Blumberg, 1992).

"Vanilloid" receptors are selectively expressed on the plasma membrane of certain primary afferents. The interaction of capsaicin with these receptors opens a voltage insensitive (ligand-gated) non-selective cation channel allowing sodium to flow down its concentration gradient into the cell causing depolarization (Maggi, 1991; Szallasi & Blumberg, 1992). This depolarization generates an afferent impulse so that Ca^{2+} enters the cell from the extracellular space leading to membrane depolarization (action potential). It has been shown that in the absence of Ca^{2+} there still is depolarization of the membrane suggesting that Na^+ may be responsible for capsaicin depolarization and impulse generation (Maggi, 1991). Depolarization of the membrane caused the release of neuropeptides from the same terminal, i.e. without an obligatory contribution of nerve conduction (Maggi, 1991). Although the role of Ca^{2+} in the excitatory action of capsaicin is unclear, Ca^{2+} influx through this channel appears to be necessary for capsaicin-induced neurotransmitter release, desensitization and neurotoxicity (Marsh et al., 1988; Maggi et al., 1989b).

Ruthenium red, an inorganic dye with the ability to block Ca^{2+} entry, protects from capsaicin desensitization (Maggi et al., 1988b). Ruthenium red selectively prevents the capsaicin-induced release of neurotransmitter from primary afferents (Maggi, 1991). Ruthenium red blocks Ca^{2+} transport not only at the cell membrane level but also in mitochondria. This intracellular site of action could be important for capsaicin desensitization of primary afferents (Jancso et al., 1984). As well, the effects of ruthenium red on capsaicin involve either antagonism at the level of capsaicin receptor, blockade of cation channel coupled with the capsaicin receptor, or combination of both (Chahl, 1989).

The secretion of peptide from capsaicin sensitive nerves is via voltage-sensitive Ca^{2+} channels (Maggi, 1991). Release of transmitters by capsaicin was not affected by ω-conotoxin, a selective blocker of N-type voltage sensitive calcium channels, but was blocked by ruthenium red (Maggi, 1991). By contrast, release of transmitter from primary afferents by other stimuli such as electrical field stimulation and high concentrations of potassium was ω-conotoxin-sensitive but, ruthenium red resistant (Maggi et al., 1989a; Maggi, 1991). These findings suggest that there may be two different ways for the release of transmitter from capsaicin-sensitive primary afferents: 1) via direct Ca^{2+} dependent; 2) via N-type voltage-sensitive calcium channels.

1.2.2. Capsaicin-induced desensitization

Capsaicin has long been known to produce initial extensive excitation of sensory neurons, followed by a prolonged neuroinhibitory action commonly referred to `capsaicin desensitization' (Maggi, 1991; O'Neill, 1991). This property has been exploited extensively as a tool with which to investigate the role of these nerves in airways.

The extent of excitation and functional or anatomical disruption of the capsaicin-sensitive neurons depends on duration and magnitude of exposure of these nerves. As well, the species, the route of application and the developmental age of animal are important factors (Maggi 1991; O'Neill, 1991). Exposure to low doses of capsaicin causes selective desensitization to capsaicin itself, but exposure to high doses of capsaicin leads to non-specific desensitization (Brugger et al., 1988; Dray et al., 1989).

Capsaicin-selective desensitization could be explained by capsaicin receptor down regulation or loss of vanilloid binding sites (Szallasi & Blumberg, 1992). Non-specific desensitization is probably due to the neurotoxic action of increased Ca^{2+} influx produced by high doses of capsaicin (Wood et al., 1988). Capsaicin induces ultrastructural changes (e.g. mitochondrial swelling via activation of Ca^{2+}-dependent enzymes) and has cytotoxic effects on primary afferents. These effects are prevented by exposure to a Ca^{2+} free medium indicating that it is Ca^{2+}-dependent (Marsh et al., 1988).

Another mechanism for the neurotoxic action of capsaicin is represented by Na^+ influx along the capsaicin-activated cation channel which leads to uptake of Na^+ and Cl^-, influx of water and osmotic lysis (Winter et al., 1990; Dray, 1992b). Depletion of the sensory neuropeptides accounts, at least in part, for a long-lasting impairment of the efferent function of capsaicin. This function recovers in parallel to recovery of tissue levels of sensory neuropeptides (Maggi et al., 1987b). Other possibilities include internalization of the receptor or down regulation of the second messenger-coupled system.

In spite of progress in our knowledge about capsaicin, still the question of "what is the physiological role of vanilloid receptors?" remains to be answered. Also the endogenous ligand for these receptors remains unknown. A possible role of these receptors may be associated with the hyperalgesia found in inflamed tissue (Bevan & Yeast, 1989). It has been shown that ruthenium red prevents primary afferent activation induced by noxious heat or application of toluene diisocyanate. These findings suggest that these stimuli may release an endogenous capsaicin-like substance(s) (acting at the capsaicin receptor) or could stimulate a distinct receptor(s) that share a common membrane transduction/ion channel with the capsaicin receptor (Amann et al., 1990; Maggi et al., 1990a).

1.2.3. Afferent-efferent dual function of capsaicin-sensitive sensory neurons

The capsaicin-sensitive neuron represents a peculiar type of sensory cell able to release stored transmitters both peripherally and in the central nervous system. The release of transmitter at different sites determines the sensory-efferent function of capsaicin (Maggi & Meli, 1988). The release of transmitter in CNS or ganglia and in the periphery represents the sensory and efferent functions, respectively.

Capsaicin acts on sensory neurons to release mediators from peripheral terminals via an axon reflex, or directly by stimulating the same terminal (Maggi & Meli, 1988). This single event causes depolarization of sensory nerve endings and secretion of transmitters in the periphery. By propagating an action potential, there can be release of transmitters both in the CNS and the periphery (Szolcsanyi, 1983; Maggi et al., 1987a).

Whether depolarization of sensory nerve endings evokes central or axonal reflex-mediated effects is controversial (Biggs & Goel, 1985; Maggi & Meli, 1988; Kroll et al., 1990). Most investigators showed that centrally-mediated reflexes had no role in the capsaicin-induced release of neuropeptides in the lungs (Gamse et al., 1979b; Saria et al., 1983; Biggs & Goel, 1985). Saria et al. (1983) showed that visceromotor responses to capsaicin are neurogenic and Na^+ channel blockade with local anesthetic or tetrodotoxin did not change these responses. However some investigators showed that at cutaneous level the inflammatory responses to capsaicin are blocked by local anesthetics suggesting the involvement of axonal reflexes (Jancso et al., 1986; Foreman & Jordan, 1984; Szolcsanyi, 1984b).

There also are conflicting views on the role of centrally mediated-reflexes in capsaicin's pulmonary effects, *in vivo.* Some have found pharmacologic evidence for central reflex effects (Delpierre et al., 1981; Ballati et al., 1992), others have found none (Szolcsanyi, 1983; Biggs & Goel, 1985; Maggi & Meli, 1988).

It is believed that SP is the mediator of the axon reflex at cutaneous levels where it causes vasodilation and plasma extravasation via mast cell degranulation (Skofitsch et al., 1985). This could not apply for certain viscera as it has been shown that tetrodotoxin and local anesthetics could not block the motor response to capsaicin (Szolcsanyi, 1983; Santicioli et al., 1986; Maggi et al., 1987a). The release of SP is believed to be the mechanism involved in a variety of visceromotor responses to capsaicin (Bartho et al., 1982; Maggi et al., 1985). However, the discovery of other neuropeptides e.g. neurokinin A (NKA), neuropeptide K and calcitonin gene-related peptide (CGRP) gave a more accurate view regarding the mechanisms involved in motor responses to capsaicin. These neuropeptides together with SP are costored and coreleased in capsaicin-sensitive sensory nerves in both the central and the peripheral nervous system (Lundberg & Saria, 1987; Martling et al., 1988).

Some of the effects of capsaicin can be attributed to the release of CGRP from sensory nerves (Franco-Cereceda & Lundberg, 1985; Saito et al., 1986). Also in some preparations (rat duodenum, uterus and vas deferens) desensitization to exogenous CGRP prevented the effects of low doses of capsaicin which suggest that these responses are mediated via endogenous release of CGRP from the sensory nerve fibres (Hua et al., 1986; Hua & Lundberg, 1986).

At high doses, capsaicin showed excitatory effects which could be explained by the release of TK. Taken as a whole, there is general agreement that at least part of capsaicin's effects are mediated by its ability to release neuropeptides from sensory nerve endings. In this regard the bronchospastic effects of capsaicin could be explained by the release of TK from the sensory nerve endings. Thus, non-selective or selective neuropeptide receptor antagonists, or the ablation of sensory nerve endings by pretreatment with large amounts of capsaicin, greatly reduced or prevented capsaicin-induced bronchospasm (Karlsson et al., 1985; Saria et al., 1985). Any discrepancies may be explained by the differences among species, organs, type of response measured and systems of measurement. The majority of the species differences have been considered as different at the level of transmitter released in the nerve terminals or in expression of NKR (Szallasi & Blumberg, 1993).

1.2.4. Heterogeneity of capsaicin-sensitive sensory neurons

The importance of capsaicin-sensitive sensory fibres in the pathogenesis of human disease such as asthma and bronchial hyperreactivity led investigators to look at the mechanisms by which environmental stimuli activate these elements. An understanding of the way natural stimuli activate these elements could help in finding drugs which modulate these functions. Capsaicin-sensitive neurons behave as polymodal receptors as various types of stimuli, physical and chemical stimuli, could activate them. There are some controversies regarding the main mechanisms of activation of these elements. Some believe that chemical signals are the major natural stimuli (Coleridge & Coleridge, 1981; Coleridge & Coleridge, 1984). Others concluded that physical stimuli e.g. distension and pulmonary edema are the major stimuli (Pintal, 1986; Roberts et al., 1986). It should be noted that physical stimuli can activate biochemical events e.g. production of prostanoids and bradykinin (Piper & Vane, 1971). Therefore, distinguishing the relative role of physical or chemical stimuli is rather more complicated than what was expected.

As a variety of stimuli can activate capsaicin-sensitive sensory neurons, these elements are probably heterogeneous (Matsuyama et al., 1986; Rose et al., 1986). Different type of sensory neurons have been detected. Although it is not very clear, some evidence showed that activation of different types of sensory neurons leads to the release of different mediators (Maggi & Meli, 1988).

1.2.5. Coexistence of neuropeptides in capsaicin-sensitive neurons

Immunohistochemical studies have shown that different neuropeptides and even non-peptide transmitters are released by capsaicin in both CNS and periphery. Among these transmitters SP, NKA, neuropeptide K, CGRP, VIP and somastatin have been most studied (Maggi & Meli, 1988). Different types of capsaicin-sensitive sensory neurons could account for this diversity. Another factor may be the intensity of stimulation. Functional studies have shown that there are some interactions among these transmitters and sometimes the effect of one transmitter is overcome by another one (Maggi & Meli, 1988); thus to get a clear picture it is necessary to measure biologic effects and measure neurotransmitter release simultaneously.

Administration of low doses of capsaicin causes bronchoconstriction through the release of neurotransmitters. However, administration of high doses of capsaicin causes a long-lasting desensitization of the sensitive primary afferent neurons, along with neuropeptide depletion (Maggi, 1991; O'Neill, 1991). These findings indicate the importance of capsaicin-sensitive afferent in airway pathophysiology, but they cannot show the relative importance of individual neuropeptides released from the primary afferent nerves.

1.3. NANC excitatory system

A noncholinergic excitatory pathway has been demonstrated in several species including human and guinea-pig (Barnes, 1991b). The main mediators for this system are SP, NKA and CGRP (Palmer & Barnes, 1987; Barnes, 1991b). Electrical stimulation of guinea-pig trachea causes atropine-resistant bronchoconstriction. This response is mimicked by SP and inhibited by SP antagonists supporting the involvement of SP as a neurotransmitter of NANC excitatory system (Lundberg et al., 1983b). SP belongs to a family of closely related peptides called TK (Maggi, 1990). Another transmitter of NANC excitatory is CGRP which is costored and coreleased with TK (Karlsson & Persson, 1989; Pendry, 1993).

1.3.1. Tachykinins

TK are a group of peptides that in mammals comprise SP, NKA (and its N-terminally altered form neuropeptide K) and neurokinin B (NKB) (Maggi, 1990). Historically, the first peptide of this group was discovered by Von Euler and Gaddum (1931) while studying the tissue distribution of ACh in equine intestine and brain. They found an extract that maintained hypotensive and spasmogenic activity on isolated rabbit jejunum in the presence of atropine. This compound was termed SP (P for purification). TK are widely distributed and active in both the CNS and the periphery (Maggi, 1993).

TK evoke a variety of biological responses, including vasodilation and paradoxical contraction of gastrointestinal, urinary and bronchial smooth muscle, mucus secretion, increased microvascular permeability and decreased mucociliary clearance (Maggio, 1988;

Nelly & Charle, 1991; Barnes, 1991a). The principal biologic activities of these peptides reside in their structurally similar carboxyl sequence, ...Phe-X-Gly-Leu-Met-COOH, where X is a branched aliphatic or aromatic amino acid. However, it has been shown that some of the effects of TK such as behavioural effects are mediated by N-terminal of these peptides, where there is considerable variability in amino acid composition (Maggi, 1990; Frossard & Advenier, 1991; Gerhardt et al., 1992; Larson & Sun, 1994). It is thise part of the molecule that accounts for the different biologic activities e.g. potency, efficacy and duration of action (Erspamer & Melchiori, 1973; Erspamer, 1981).

TK are synthesized in nerve cells and released locally, possibly as a result of local reflexes (Gamse et al., 1979a; Nelly & Charles, 1991) and produce their biological effects via activation of specific receptors (Nelly & Charles, 1991).

1.3.1.1. TK and their expression

SP can be encoded by 3 precursors, α, β and γ-preprotachykinin mRNAs. NKA can be encoded by both β and γ-preprotachykinin mRNAs (Nelly & Charles, 1991). cDNA encoding for NKB has been identified in rat cerebral cortex and appears to be produced by neurons different from those containing the precursors for SP and NKA (Nelly & Charles, 1991).

1.3.1.2. TK receptors

TK effects are mediated via activation of neurokinin receptors (NKR). So far, three NKR have been found; NK1, NK2 and NK3 receptors (Maggi, 1990; Hiroki & Shigetada, 1991; Nelly & Charles, 1991; Krause et al., 1992). Figure 1.4 shows the structure of NK1 receptor. Features common to all members of this family of receptors include the presence of seven hydrophobic protein sequences, each of which is believed to span the cell membrane and interact with one or more GTP-binding proteins to promote high affinity binding with ligand and transduce intracellular signals (Dohlman et al, 1987; Krause et al., 1992). These receptor molecules appear to be involved in promoting transcellular communication with the environment (Cascieri & Liang, 1983; Coats & Gerard, 1989). Common sequence motifs among the TK allow them to interact with all of the receptors, but with different affinity (Buck et al., 1984; Burcer et al., 1986). The NK1 receptor is selective for SP > NKA > NKB; the NK2 receptor binds NKA > SP > NKB; the NK3 receptor is selective for NKB > NKA > SP (Nelly & Charles, 1991; Krause et al., 1992). As a result of this cross reactivity, dissection of the ligand-receptor responsible for particular physiological responses *in vivo* has been somewhat difficult. However, the molecular cloning of the TK receptor gene and cDNAs from several species have provided valuable information relating to the structure of these molecules. Also, development of selective agonists and antagonists provides additional information about the ligand binding sites and structure-function relationships that are involved in signal transduction, tissue localization and the regulation of expression of these receptor molecules.

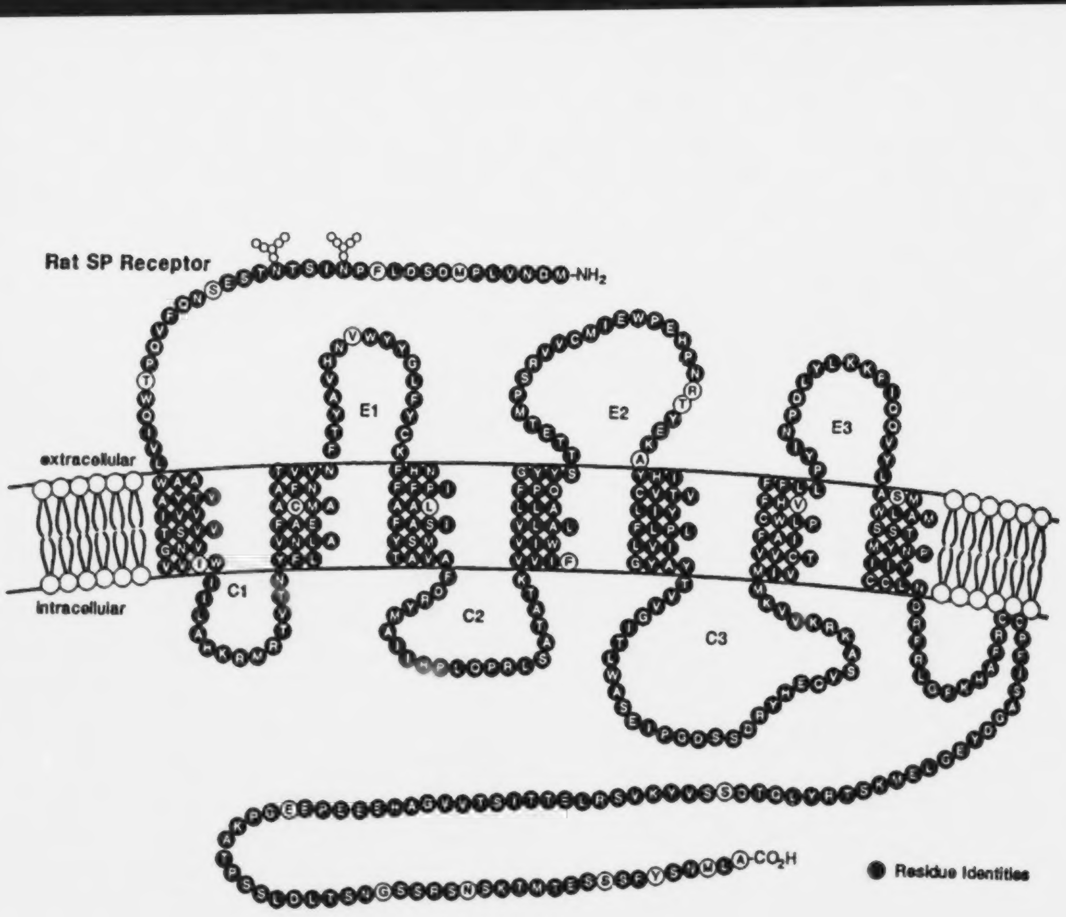

Rat SP Receptor
-NH₂
extracellular
intracellular
E1
E2
E3
C1
C2
C3
-CO₂H
● Residue Identities

Fig 1.4 (from Krause et al., 1992)

Primary structure of the the NK1 receptor. Rat and human NK1 or substance P receptor structures are shown in a two-dimensional topographic illustration, whereby the seven putative α-helical transmembrane domains divide the sequences into extracellular and intracellular domains. Consequently, the amino terminal domain is orientated extracellulary, as extracellular domains 1-3 (E1, E2 and E3), and the remaining domains (cytoplasmic domains C1, C2 and C3 and the carboxy terminal region) are oriented toward the cytoplasm. Residue identities are shown as darkened circles and the clear circles represent the rat sequence. Clear circles on the peptide chain without any letters represent the branched glycan chains.

1.3.1.2.1. Structural characterization of the NKR

Expression cloning in *Xenopus* oocytes, hydropathy analyses and pharmacological evidence revealed seven hydrophobic membrane-spanning sequences for these receptors which suggests TK signalling is mediated by activation of GTP-binding proteins (Lundberg & Saria, 1987; Manzini et al., 1987; Gerard et al., 1990; Gerard et al., 1993). cDNA cloning and structural studies show high homology among these receptors. However, there are differences in potential glycosylation sites which may account for some of the pharmacologic difference among species (Abdel-Latiff, 1986). Activation of the receptor was shown to result in the rapid and transient appearance of inositol-1,4,5-trisphosphate (IP3) (Payan et al., 1986; Krause et al., 1992). By crosslinking radiolabelled SP and NKA to their receptors and doing SDS-PAGE, several specifically labelled proteins with different molecular weights were observed (Payan et al., 1984; Payan et al., 1986; Coats & Gerard, 1989). These findings together with pharmacological evidence suggest that there might be multiple receptor subtypes (Quirion et al., 1991). This so-called "receptor family" is coupled to and contains seven hydrophobic α-helical transmembrane domains, with an extracellular amino terminus and intracellular carboxyl terminus (Fig. 1.4).

1.3.1.2.2. Distribution of NKR

A few years ago, most of the studies to analyze functional properties and tissue distribution of the TK were based on the use of functional cDNA clones for each of the three NKR, as selective pure antagonists, specific radioligands and specific antibodies were not readily available. It was shown that mRNA for NK1 receptors was distributed both in the nervous system and peripheral tissues. mRNA for NK3 receptors was also distributed in various tissues, but it was expressed more predominantly in the nervous system than in peripheral tissues. By contrast, mRNA for NK2 receptors was localized in peripheral tissues (Hiroki & Shigetada, 1991; Gerard et al., 1993).

The development of selective and potent agonists and antagonists facilitated the characterization of the structural and biochemical features and tissue distribution of NKR. For NK1 receptor binding studies [^{3}H]-[Sar^9,Met(O)^{11}]-SP and [^{125}I]BH-[Sar^9,Met(O)^{11}]-SP, highly selective NK1 receptor radioligands, have been used (Quirion et al., 1991). Studies using these agonists suggested the possible existence of NK1 receptor subtypes (Quirion et al., 1991).

Overall, studies using selective agonists, cDNA cloning, sequence analysis and functional expression show that NK1 receptors are expressed by neurons and glia in the CNS, neurons within the myenteric plexus, smooth muscle cells, endothelial cells, fibroblasts and various types of circulating immune and inflammation-activated immune cells (Krause et al., 1992). The NK2 receptors are primarily expressed in peripheral tissues (smooth muscle cells) with little overlap with NK1 receptor sites (Krause et al., 1992). The NK3 receptors are primarily expressed in CNS and certain peripheral tissues, for example smooth muscle cells and portal vein, with little overlap with NK1 receptors (Krause et al., 1992).

1.3.1.3. Enzymatic degradation of TK in the airways

The effects of TK are limited by enzymatic degradation by neutral endopeptidase (NEP), angiotensin-converting enzyme (ACE), dipeptidyl (amino) peptidase and chymase (Caughey et al., 1988; Frossard & Advenier, 1991; Wang et al., 1991). NEP is present in tracheobronchial epithelial cells, in airway smooth muscle and around submucosal glands and bronchial vessels (Picard et al., 1993; Qian et al., 1993). NEP cleaves peptides preferentially at the amino-terminal site of the hydrophobic amino acid residues Phe, Leu, Ile and Val (Turner et al., 1985; Lilly et al., 1993). Pretreatment of tissues with specific NEP inhibitors leads to potentiation of the effects of the TK, *in vivo* and *in vitro* (Thompson & Sheppard, 1988; Frossard & Advenier, 1991; Opgenorth et al., 1992; Shore et al., 1992; Crimi et al., 1994).

SP is also cleaved by ACE (Frossard & Advenier, 1991, Lilly, 1993). ACE is localised mainly on the surface of vascular endothelium (Kundu & Wilson, 1992). ACE hydrolyses SP at the Phe^{8}-Gly^{9} and Gly^{9}-Leu^{10} bonds which leads to inactivation of SP (Shore & Drazen, 1988; Umeno et al., 1992; Lilly, 1993). The physiologic importance of NEP and ACE as regulators of the pulmonary actions of SP appears to depend on the route of administration of the peptide to the lung. When SP is presented to the lung via the vasculature, its airway effects are enhanced by the inhibition of ACE (Drazen et al., 1989; Lilly, 1993). The further enhancement of SP-induced pulmonary constriction when a NEP inhibitor is added to the ACE inhibitor indicates modulation of this process by NEP. NEP inhibition with phosphoramidon or thiorphan, but without captopril, augments SP-induced increases in lung resistance in anesthetized, mechanically ventilated guinea-pigs (Thompson & Sheppard, 1988). However, when SCH32615 was used to inhibit NEP, the SP response was not augmented unless ACE is also inhibited (Shore et al, 1992). Since SCH32615 is a very specific NEP inhibitor (Chipkin et al., 1988) (i.e., has no significant effect on ACE) while thiorphan has some anti-ACE activity, it appears that NEP and ACE are physiologically competitive in their capacity to degrade SP.

In contrast, when SP is presented via the airway lumen, NEP alone appears to be important in modulating its bronchoconstrictor activity. Addition of the NEP inhibitor thiorphan or SCH32615 alone to the perfusate of isolated tracheally perfused lungs increased the bronchoconstriction induced by a bolus of SP added to perfusate (Lilly et al., 1993).

Cleavage of SP by dipeptidyl aminopeptidase results in production of SP^{3-11} and SP^{5-11} which are more potent bronchoconstrictors than SP (Shore & Drazen, 1988; Wang et al., 1991). Differences among the relative potencies of TK fragments and higher potency of NK2 receptor agonists led to speculation that different NKR may be involved in these phenomena. To address this idea, we used selective NKR antagonists and characterized SP and SP-fragments-induced bronchospasm in anesthetized, mechanically ventilated guinea-pigs.

1.3.1.4. Mechanisms of action of TK

In a number of tissue preparations, stimulation of NKR induces hydrolysis of phosphatidyl inositol (PI) (Manthy et al., 1984; Hunter et al., 1985; Johnson et al., 1991). In some preparations, TK also enhanced synthesis of cAMP (Hunter et al., 1991).

The discovery of several non-peptide NKR antagonists promises to be useful for binding site determination as well as for pharmacologic studies. Interaction of NKR with TK activates a G-protein which in turn stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) via the activation of phospholipase C (PLC). This causes the production of two second messengers, water soluble (IP_3) and lipid soluble diacylglycerol (DAG) (Abdel-Latiff, 1986). IP_3 diffuses from the plasma membrane to the endoplasmic reticulum (ER), where it binds to IP_3 receptors which control Ca^{2+} release and thus mobilizes Ca^{2+} from this subcellular organelle (Abdel-Latiff, 1986). The release of Ca^{2+} from the ER into the cytoplasm results in the initial raise in Ca^{2+} concentration, which in smooth muscle couples the agonist-receptor interaction to the fast component of the contraction response. Excretion of Ca^{2+} from the ER by IP_3 signals the influx of extracellular Ca^{2+}. Although the influx of extracellular Ca^{2+} is not required for the initial phase elevation of Ca^{2+} concentration, it is required for maintenance of the elevated Ca^{2+} concentration which is necessary for a maximal and sustained response and to reload the Ca^{2+} stores (Abdel-Latiff, 1986). Ca^{2+} is required for many intra- and extra-cellular functions such as muscle contraction, cell membrane stability, etc. (Abdel-Latiff, 1986; Walsh, 1991). Figure 1.5 shows the mechanism by which cellular Ca^{2+} is regulated by PLC-linked NK1 receptor in exocrine gland cells.

1.3.1.5. TK and airway pathophysiology

TK, particularly SP and NKA, are widely distributed in the airways and lungs of several species (Lundberg et al., 1983a; Lundberg & Saria, 1987). The major source of

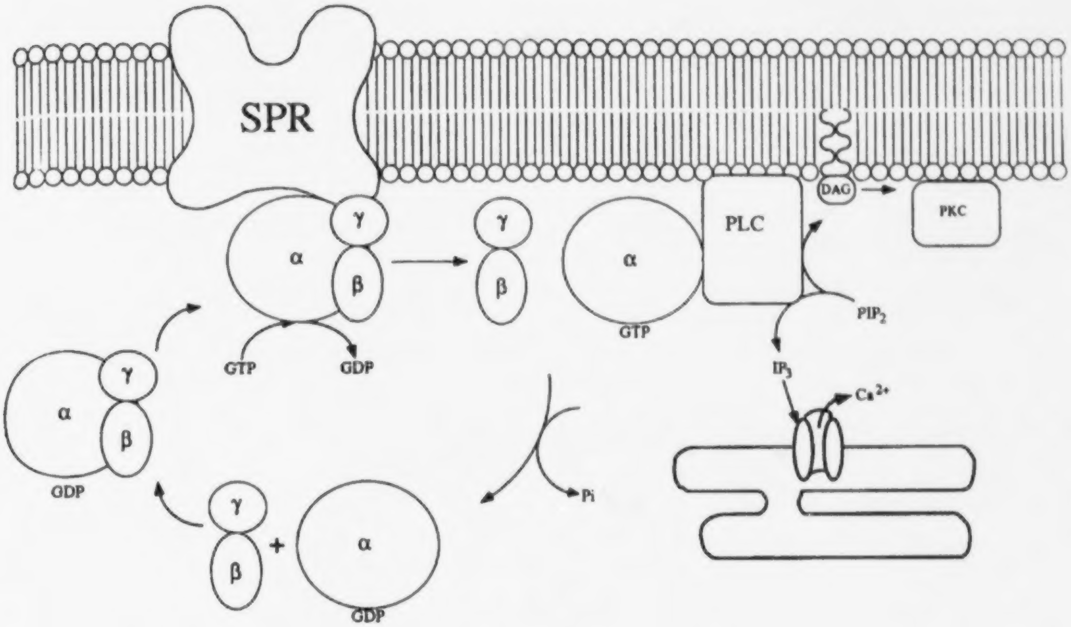


Fig 1.5 (from Krause et al., 1992)

A model for the mechanism of action of substance P acting on the NK1 receptor. Ligand stimulation of the receptor activates a G-protein cascade in mammalian cells. Dissociation of Gα from the G-protein complex due to ligand stimulation results in the activation of PLC by a pertussis toxin insensitive mechanism. PLC catalyses the breakdown of inositol-containing phospholipids and generates two second messengers, the calcium mobilizing agent inositol 1,4,5-triphosphate and an activator of protein kinase C, diacylglycerol.

TK in the airways is the peripheral endings of certain primary afferent neurons which are sensitive to the stimulant and toxic actions of capsaicin (Szolcsanyi, 1984a, Maggi & Meli, 1988; Fuller, 1990). The preprotachykinin I gene is expressed by capsaicin-sensitive afferent neurons innervating the mammalian airways. This indicates that SP and NKA are costored and coreleased from sensory nerve terminals in the airways (Lundberg & Saria, 1987; Maggi, 1990). It has been shown that the capsaicin-sensitive afferents play a dual, sensory and efferent function. In the airways, the sensory function of these nerves deals with a number of reflex responses, including cough to inhaled irritants (Barnes, 1986a). The efferent function involves a variety of biologic effects, including bronchoconstriction, vasodilation, increase in plasma protein extravasation, recruitment of inflammatory cells etc. (Maggio, 1988; Nelly & Charle, 1991; Barnes, 1991a). These effects comprise neurogenic inflammation.

Because of the presence of TK in the airways and their ability to mimic the various pathophysiological feature of asthma, SP and NKA are considered as possible mediators in asthma. Data from several laboratories support the role of neurogenic inflammation in animal models of asthma/bronchial hyperreactivity (Barnes, 1986a; Lundberg & Saria, 1987; Maggi,1990; Ichinose et al., 1993). Also, studies on autopsy tissue, bronchoalveolar lavage and sputum suggest that in asthma the SP content of airways may be increased (Joos et al., 1994). Interestingly, Adcock et al. (1993) showed that there was an increase in NK1 receptor mRNA in the asthmatic lungs compared with non-asthmatic control tissues. By contrast, they observed no change in the NK2 receptor mRNA expression. This increase in NK1 receptor gene expression may cause an increase in the NK1 receptor numbers and contribute to inflammatory actions of TK in the lung.

It was shown that SP is the most potent TK to induce plasma protein extravasation, which leads to the conclusion that NK1 receptors are involved in plasma protein extravasation (Maggi, 1990). Also using selective NK1 and NK2 receptor antagonists, Tousignant et al. (1993a; 1993b) showed that plasma protein extravasation in guinea-pig trachea and large airways was mediated by NK1 receptors but that in lower airways NK2 receptors were responsible for this effect. The C-terminal of TK is responsible for activation of NK1 receptors (Maggi, 1990; Frossard & Advenier, 1991). However, in guinea-pig lower airways, there is evidence for the presence of NK2 receptor mechanisms mediating plasma protein extravasation (Tousignant et al., 1993a).

TK induce bronchoconstriction via NK2 and to a lesser extent via NK1 receptors. There are considerable differences among species regarding the involvement of NKR. Thus in guinea-pigs both NK1 and NK2 receptors mediate bronchoconstriction however, in humans NK2 receptors mediate bronchoconstriction (Ellis, 1995: Lundberg, 1995). The C-terminal of TK is involved in activation of NK2 receptors (Regoli et al., 1990; Cascieri et al., 1992). SP also appears to release endothelium-derived relaxant factor (EDRF) as well as prostaglandin E_2 (PGE_2) from airway epithelium via NK1 receptors on epithelial cells (Barnes, 1991a). EDRF and PGE_2 counteract the constrictor activity of SP on airway smooth muscle. Epithelium removal markedly potentiated the bronchoconstrictor effect of TK (Barnes, 1991a). This could be due to loss of EDRF, PGE_2 and NEP.

1.3.1.6. TK and pulmonary circulation

Capsaicin-sensitive nerves in blood vessels contain SP, NKA and CGRP. These transmitters have potent vasodilatory effects (Maggi, 1990). TK produce an endothelium-dependent vasodilation of the pulmonary artery. The endothelium-dependent vasodilation involves activation of NK1 receptors (Maggi, 1990). Removal of the vascular endothelium leads to vasoconstrictor effects of TK via NK2 receptors (Maggi, 1990). This paradoxical effect of TK on the vasculature (endothelium-dependent vasodilation and vasoconstriction in rubbed vessels) still remains to be understood.

1.3.1.7. TK and mucus secretion

Although the major mechanism mediating neurogenic mucus secretion is cholinergic, a significant secretory response is mediated by NANC via TK (Rogers, 1995). NK1 receptors mediate mucus secretion from both seromucus glands and goblet cells. The idea of involvement of NK1 receptor in mucus secretion was supported by the use of selective NK1 receptor agonists and antagonists (Kuo et al., 1990; Geppetti et al., 1992).

1.3.1.8. TK and cells of inflammation

Inflammation is a dominant pathophysiological feature of the asthma/bronchial hyperreactivity. TK exert a variety of biological actions on inflammatory cells. Although some of these cells e.g. mast cells and alveolar macrophages are normally present in the lung, other cells e.g. granulocytes, lymphocytes and eosinophils migrate in the inflamed tissue (Maggi, 1990). There is some evidence that specific NKR are present on several types of inflammatory cells (Joos & Pauwels, 1990; Frossard & Advenier, 1991). Von Essen et al. (1992) showed that pM concentrations of TK stimulate the release of neutrophil chemotactic factor(s) from bovine airway epithelial cells in culture. The effectiveness of low concentrations of TK in this study suggests that this effect is mediated via TK receptors. NK1 receptors are involved in this response (Frossard & Advenier, 1991). It was shown that SP and NKA increased proliferation of human T-lymphocytes at nM and pM concentration, respectively (Maggi, 1990). The greater activity of NKA suggests the involvement of NK2 receptors. Brunelleschi et al. (1992) showed that TK stimulate guinea-pig alveolar macrophages via activation of NK1 receptors. SP slowed PMN and eosinophil infiltration via mast cell degranulation. However NKA and kassinin, which have high affinity for NK2 and NK3 receptors, respectively, were inactive (Maggi, 1990). NKA aerosol exposure causes neutrophil recruitment (Kudlacz & Knippenberg, 1994).

1.3.1.9. TK and airway responses to antigen

Various studies suggest activation of sensory nerves during allergic reactions in the airways. Pretreatment with capsaicin attenuates allergic reactions to acute exposure to antigen (Joos & Pauwels, 1990). Ellis and Undem (1992a) reported that antigen application enhanced TK-mediated atropine resistant contractions produced by electrical stimulation of sensory nerves. Giving NKR antagonists abolished this response, but it did not modify the response to exogenous SP. Furthermore, they showed that antigen-potentiated contractions were blocked by pyrilamine. They concluded that antigen stimulation of mast cells released histamine that facilitated TK release.

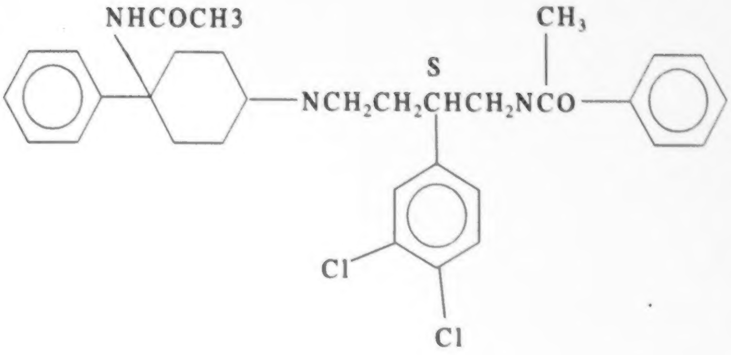
1.3.1.10. NKR antagonists

Capsaicin has been used to examine the involvement of TK in the pathophysiology of asthma. Using capsaicin enables one to look at the importance of capsaicin-sensitive afferents in airway pathophysiology, but it cannot show the relative importance of individual neuropeptides released from the primary afferent nerves. To overcome the problem in determining which types of the NKR are involved in the specific effect, selective NKR antagonists are available.

From the early 1980s, much interest was on production of NKR antagonists. The best example of the first generation of NKR antagonists was the undecapeptide SP derivative, spantide I (Maggi, 1993). Due to low potency and limitations linked to their peptide nature, these peptides have not been considered as good candidates for these studies. The second generation of NKR antagonists showed high potency and selectivity for only one of the three NKR (Maggi, 1993). Problems with these antagonists were associated with limitations linked to their peptide nature and their long duration of action in vivo (Maggi et al., 1993).

Finally the third generation of NKR antagonists, nonpeptide ligands, which are potent and highly selective for NK1 (CP 96,345, CP 99,994 or RP 67,580) and for NK2 (SR 48,960) receptors were discovered (Advenier et al., 1992; Martin et al., 1992; Picard et al, 1993; Qian et al., 1993). The chemical structures of some of NKR antagonists are given in Figure 1.6.

SR 48,968


NHCOCH3
CH3
S
NCH2CH2CHCH2NCO
Cl
Cl

CP 96,345

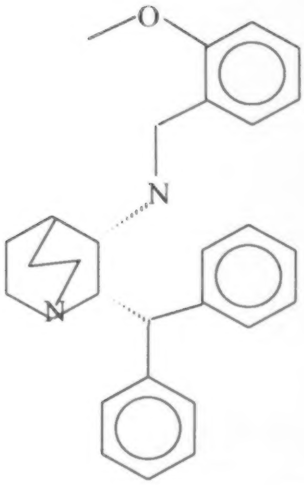


Fig. 1.6

Chemical structures of non-peptide neurokinin receptor antagonists 48,968 and CP 96,345.

The use of second and third generation of NKR antagonists helped investigators make firm conclusions about involvement of NKR in the pathophysiology of the airways. Atropine-resistant bronchoconstriction is produced in guinea-pig airways by nerve stimulation via TK release from sensory nerves. When exogenous TK were used, it was shown that both NK1 and NK2 receptors were involved in this effect. However, endogenously released TK generated by stimulating sensory nerves causes bronchoconstriction mainly via NK2 receptors (Maggi et al, 1991a). It should be noted that there are marked differences among species regarding the role of different NKR (Belvisi et al., 1994). Stimulation of the vagi, antigen challenge and administration of mediators of inflammation cause TK release that produced increases in plasma extravasation. This is mediated through only NK1 receptors (Maggi et al, 1991a; Tousignant et al., 1993; Nicolau et al., 1993). Also, it was shown that NK1 receptors are responsible for increased mucus secretion by inhaled TK in the airways (Von-Essen et al., 1992). These studies led investigators to the idea that the development of potent and selective TK antagonists might lead to a class of novel drugs with therapeutic potential for the treatment of asthma and/or bronchial hyperractivity.

1.3.2. CGRP

CGRP, an endogenous 37 amino acid containing peptide, belongs to a family of peptides comprising calcitonin, katacalcin and CGRP (Zaidi et al., 1990b). CGRP is a product of alternative processing of RNA transcripts of the calcitonin gene (Nakamuta et al., 1986). Four CGRP sequences have been detected from which two structurally related forms; α- and β-CGRP seem to be present in mammalian tissue (Steenbergh et al., 1985; Zaidi et al., 1990a). The distribution of α- and β-CGRP is different but, they behave similarly (Morris et al., 1984; Zaidi et al., 1990a). The amino acid sequence of CGRP is well conserved among species which indicates its importance as a vasoactive peptide (Zaidi et al., 1990b). CGRP is present in a variety of neurons in the CNS and periphery (Rosenfeld et al., 1983; Lundberg et al., 1992). In sensory neurons, CGRP is mostly colocalized with TK (Lundberg et al., 1985). CGRP immunoreactive sensory neurons are present within lining epithelia, around blood vessels and non-vascular smooth muscle and the myocardium of the atria (Lundberg et al., 1992).

1.3.2.1. Origin and secretion of CGRP

CGRP has been detected immunochemically in thyroid, pituitary, brain, gastric nerves and genitourinary tract (Clague et al., 1985; Steenbergh et al., 1985; Santicioli et al., 1988). CGRP is found abundantly in the nervous tissue, especially in the cardiovascular system (Zaidi et al., 1987a). However, abundant CGRP-specific binding sites were found not only in the nervous system but also in some peripheral tissues such as spleen, liver and lung (Nakamuta et al., 1986). CGRP-like immunoreactivity was observed in nerves from the epiglottis down to peripheral bronchi in rat, cat and guinea-pig and also in human bronchi (Martling et al., 1988). Circulating CGRP is derived mainly from perivascular and cardiac nerve terminals (Zaidi et al., 1990a). However, it is not certain that the sole source of CGRP is the nerve terminals.

CGRP is released upon activation of peripheral branches of sensory neurons by capsaicin, bradykinin, high potassium concentrations, antidromic nerve stimulation, nicotine and ouabain (Martling et al., 1988; Franco-Cereceda et al., 1989; Lou et al., 1991). Release of CGRP by these stimuli is Ca^{2+}-dependent indicating exocytosis from storage vesicles (Franco-Cereceda et al., 1989; Lundberg et al., 1992).

1.3.2.2. Metabolism of CGRP

It is likely that CGRP is inactivated by enzymatic degradation as phosphoramidon, a NEP inhibitor, has been shown to increase CGRP overflow from the isolated guinea-pig lung (LeGreves et al., 1989; Kroll et al., 1990). By contrast, some investigators showed that it is protected from plasma peptidases (Brain & Williams, 1985). Interestingly, it was shown that the cardiovascular effects of CGRP persist for a long time even after its plasma levels have fallen to normal (Struthers et al., 1986; Benjamin et al., 1987; Adnot et al., 1991). This could be explained by slow rate of metabolism or slows dissociation of CGRP from its receptor.

1.3.2.3. CGRP receptors

There is a family of receptors for CGRP in the nervous system. The highest number of high-affinity CGRP-specific binding sites have been found in cerebrum, spinal cord and other brain regions suggesting widespread involvement of CGRP in a variety of brain functions. CGRP receptors are also found in membranes prepared from lung (Zaidi et al., 1990a).

Most of the effects of CGRP are via CGRP receptors while some of them are mediated via calcitonin receptors (Zaidi et al., 1987a). Some of the effects of CGRP appear to be mediated by cAMP (Zaidi et al., 1990a). The involvement of the different regions of CGRP in binding to CGRP receptors is not clear. Thus, Chiba et al. (1989) showed that the carboxy terminal of CGRP, CGRP$^{8-37}$, was able to bind to CGRP and calcitonin receptors but that it antagonised the effects of CGRP. These findings together with those of others indicate that amino terminal region of CGRP, especially the Cys^{2}-Cys^{7} disulfide bridge, is essential for inducing subsequent intracellular signal transduction (Seifert et al., 1985). However, Maggi et al., (1990c) showed that N-terminal fragments of CGRP had biological activities similar to intact CGRP, but they were 100-1000 times less potent than intact CGRP. The lower potency of N-terminal fragments of CGRP suggests that the middle and C-terminal regions are important for maximal peptide-receptor interaction. This idea is confirmed by the findings of Shaw et al. (1992) who used MAb against different regions of CGRP and concluded that the active site of CGRP was located in the N-terminal but that the C-terminal or middle region of the peptide played an important role in determining the structural conformation necessary for peptide-receptor coupling. However, in some preparations, it was shown that the integrity of the N-terminal disulfide bridge was not critical for maintenance of the biologic activities of CGRP (Quirion et al., 1992).

Using CGRP$^{8-37}$ and CGRP$^{12-37}$, CGRP antagonists, in different experiments indicated the existence of CGRP receptor subtypes (Quirion et al., 1992). Two distinct receptor subtypes, CGRP$_1$ and CGRP$_2$, for CGRP have been reported (Dennis et al., 1990). $CGRP_1$ receptors are sensitive to $CGRP^{8-37}$ while $CGRP_2$ receptors are unaffected by this antagonist (Bartho et al., 1993). These receptors showed little or low affinity for calcitonin-like peptides (Quirion et al., 1992). Another CGRP receptor subtype has been reported in the brain (Dennis et al., 1991).

1.3.2.4. Biologic effects of CGRP

CGRP induces a variety of biologic effects including modulation of nicotinic receptor activities at the neuromuscular junction, reduction of gastric acid secretion, peripheral blood vessel dilation, cardiac acceleration, regulation of calcium metabolism and insulin secretion, increases in body temperature, decreases in food intake and increase in somatostatin release (Grunditz et al., 1986; Okimura et al., 1986; Tshikawa et al., 1988; Miles et al., 1989; Dennis et al., 1990; Hermansen & Ahren, 1990; Adnot et al., 1991; Ren et al., 1992).

CGRP is one of the most powerful vasodilator agents with a prolonged effect (Adnot et al., 1991). The mechanism of this action is controversial. Thus, in some systems, CGRP induced endothelium-dependent vasodilation (Brain et al., 1985), but in others it showed a direct effect on vascular smooth muscle (McCulloch et al., 1986; Franco-Cereceda, 1991). This may be due to interspecies and/or organ-specific differences. Ohhashi and Jacobowitz (1985) showed that CGRP also acts on presynaptic receptors and causes inhibition of noradrenaline release. However, this was disputed by Al-Kazwini et al. (1986) who showed that inhibitory effects of human α-CGRP were not antagonized by propranolol. Also, they showed that rat α-CGRP and human α-CGRP did not alter [^{3}H]-noradrenaline uptake into the mouse vas deferens. These findings together with those of Cadieux et al. (1990) suggest that although CGRP inhibited contractor responses of mouse vas deferens, it was not by interfering with adrenergic mechanisms.

Antidromic stimulation of vagus nerve causes extravasation of plasma protein which leads to neurogenic inflammation (Krause et al., 1992). Although TK are the principle mediator of neurogenic inflammation, CGRP may play a role in this response. It was shown that CGRP potentiated the effects of TK on plasma extravasation suggesting that CGRP may play a role in pathogenesis of asthma and airway diseases (Gamse & Saria, 1985).

Although the physiological significance of CGRP in lung is still unclear, the close association of CGRP-positive nerves with smooth muscle of tracheobronchial tree suggests that this peptide may be involved in regulation of bronchomotor tone. However, whether CGRP plays a role in capsaicin's bronchospastic effects is unclear. Although CGRP has no effect on guinea-pig airway smooth muscle, *in vitro* (Martling et al., 1988; Luts et al., 1990; Parsons et al., 1992) in many tissues, there is good evidence that CGRP has neuromodulatory as well as direct effects (Cadieux et al. 1990; Cadieux & Lanoue 1990). Lundberg et al. (1985) and Martling et al. (1988) showed that CGRP had no contractile or relaxant effect in guinea-pig trachea and hilar bronchi, whereas Palmer et al. (1987a) and Hamel & Ford-Hutchison (1988) claimed that CGRP is a potent contractile agent on human airway smooth muscle and guinea-pig trachea, respectively. By contrast, some investigators showed that CGRP modulated bronchoconstriction induced by other bronchospastic agents such as carbamylcholine and 5HT (Cadieux et al., 1990). We determined the effects of hCGRP and the CGRP antagonist, CGRP$^{8-37}$, on SP-, NKA- and capsaicin-induced pulmonary responses.

1.4. Methods of assessment of airway's responsiveness

Airways' hyperresponsiveness, extreme sensitivity of the airways to physiological, chemical and pharmacological stimuli, is one of the characteristic features of asthma (Curry, 1946).

1.4.1. Direct measurement of smooth muscle function

Many studies of dose-response relationships (especially in laboratory animals) have been performed in various organs, using isolated smooth muscle preparations. These *in vitro* methods allow quantitative measurements of smooth muscle contraction as well as control of variables affecting bronchomotor tone. The length of the muscle used for this technique is important as the active tension developed in muscle depends on it (Stephens et al., 1968). Studies are usually done at or near the length at which stimulation produces a maximal response. Under these conditions, a sigmoidal curve relating the dose of agonist to the tension developed is obtained.

1.4.2. Indirect measurement of smooth muscle function

In *in vivo* studies, smooth muscle contraction must be inferred from measurements that only indirectly reflect airway caliber. In a few studies, direct measurement of airway caliber has been done by morphological methods such as bronchography (Nadel et al., 1968; Nadel et al., 1971). However, in most studies, functional measurements such as maximal flow or airway resistance, which reflect airway caliber indirectly, have been used. It should be noted that changes in structures other than the airways may influence these measurements. For example, in humans and spontaneously breathing animals, laryngeal narrowing which can account for part of the total airflow resistance may be considered as bronchoconstriction (Stransky et al., 1973; Szereda-Przestaszewska & Widdicombe, 1973).

Another factor which can affect smooth muscle contraction is lung volume, especially when airway smooth muscle tone is increased (Briscoe & DuBois, 1958). Taking this in account, comparison of airway resistance or maximal flow should be done at the same lung volume. At high lung volumes, analyzing changes in maximal airflow is complicated by the effects on airway smooth muscle tone of inhalation to total lung capacity (Green & Mead, 1974). Measurement of airway resistance or conductance provides a more sensitive specific index of the caliber of the central airways, but it is insensitive to changes in small, peripheral airways (Hahn & Nadel, 1979).

Airways' hyperresponsiveness (AHR) is demonstrated clinically by comparing asthmatics' responses to inhaled aerosols of bronchoconstrictors with those of non-asthmatic controls (ATS, 1987). It should be noted that the method of giving bronchoconstrictors also influences the response of airway smooth muscle. Common routes of delivering bronchoconstrictor drugs to airways are parenteral and aerosol or topical application. When drugs are given as aerosols, the site of deposition depends on the size of the aerosol particles. For example, fine aerosols of histamine preferentially constrict bronchioles but have no detectable effect on bronchi (Nadel et al., 1966). Timing of the delivery also affects deposition of drugs so that the bronchoconstrictor activity of drugs will be different (Ruffin et al., 1978). A disadvantage of using inhaled aerosols of agonist is that it precludes measuring responsiveness to several agonists in the same animal. Also the amount of inhaled agonist actually reaching the lower airways is unknown, because constriction of the upper airways prevents agonist from reaching to the lower airways.

Parental routes also have some restrictions. Some of the bronchoconstrictors when given parenterally cause the release of catecholamines from the adrenal medulla which may decrease the effect of the drug on airway smooth muscle (Brody & Kadowits, 1974; Ploy Song Sang et al., 1978). Inactivation of drugs during their passage through the lung is another problem associated with iv administration (Ferreira & Vane, 1967). Thus, inhaled but not iv administration of prostaglandin $F_{2\alpha}$ induces bronchoconstriction (Brown et al., 1978).

The influence of bronchoconstrictors on small or large airways depends on the route of administration. Peripheral airways are predominately perfused by the pulmonary circulation, whereas large airways are perfused by the bronchial arteries (Nadel et al., 1964). Thus, delivery of drugs into the pulmonary circulation may have different effects on the airways' caliber than when it is delivered into the bronchial circulation or inhaled as an aerosol of large particle size, and deposited in the central airways. It has been shown that iv injection of histamine constricts peripheral airways whereas histamine delivered into the bronchial arteries causes constriction of central airways (Colebatch et al., 1966).

Anesthesia is another important factor which affects airway smooth muscle responses. It has been shown that general anesthesia changes baseline airway caliber (Brenstine et al., 1957), inhibits histamine-induced bronchoconstriction (Douglas et al., 1972) and releases catecholamines (Spriggs, 1965). Some anesthetics may induce metabolic acidosis which can selectively inhibit the bronchoconstriction induced by certain drugs, e.g., 5HT (Douckles et al., 1974). The effects of anesthetics may explain some of the discrepancies among studies of the role of reflexes in bronchial reactivity.

1.5. Antibodies as tools for studying mechanisms of action of drugs or endogenous mediators

Two approaches have been employed for studying the structure-activity relationship and mechanism of action of drugs or endogenous mediators (Shaw et al., 1992). In conventional pharmacological approaches, the structure-activity relationship is studied using selective receptor agonists and antagonists or in case of endogenous peptides using sequence analogues of peptides. However, to use sequence analogues one has to do amino acid substitution, deletion and insertion which may change the tertiary structure of peptide and leads to loss of function of peptides (Shaw et al., 1992).

An alternative approach to pharmacological blockade is the use of antibodies which bind to and inhibit the biological activities of drugs or endogenous peptides. Immunoblockade may be a more general approach to investigate the physiological role of neuropeptides as no assumptions have to be made concerning receptor multiplicity and the relative selectivity of receptor antagonists. The use of MAb enables us to raise Ab directed against specific region of molecules so that it is possible to get information regarding the active sites of molecules.

1.5.1. Monoclonal antibodies (MAb)

Serum contains many different types of Abs that are specific for different antigens (Ag). The amount of specific Ab for an Ag is very low and even in a hyperimmune animal it is less than one-tenth of the circulating Ab (Abbas et al., 1994). Using polyclonal Ab creates variety of different problems for immunochemical or pharmacological studies. To overcome these problems, homogeneous Ab with predetermined specificity are needed. The technology of hybridoma production enable us to produce such Ab.

First attempts for isolation of a homogeneous population of Ab were by growing B-cell tumors in animals or tissue culture. Each B-lymphocyte produced Ab of single specificity so that this technique allowed production of a homogeneous population of Ab (Abbas et al., 1994). However, it was impractical to isolate B-cells secreting Ab of predefined specificity. B-lymphocytes are the source of production of Ab but, they can not grow *in vitro*. To overcome these problems, Kohler and Milstein (1975) developed techniques which allow the growth of cells secreting Ab with a predetermined specificity. In this technique an Ab secreting cell (B-cell), isolated from an immunized animal, is fused with an immortal myeloma cell, a type of B-cell tumour. The resulting hybrid cells are called hybridomas and have the ability to grow in tissue culture and produce Ab with defined specificity (MAb) (Eshar, 1985). When B-lymphocytes are fused with myeloma cells from different species, the resulting cells are called heterohybridomas.

1.5.2. Production of MAb

Three common technologies used to grow hybridomas are the mouse ascites technique, the conventional flask or roller bottle technique and the bioreactor (artificial capillary system). Hybridomas in conventional cell culture systems e.g. flasks, suspension cultures, or roller bottles are exposed to decreasing concentration of nutrients and oxygen and with accumulation of increasing concentrations of lactic acid and ammonium ions. In contrast, artificial capillary systems provide continuous supplies of fresh medium and oxygen and at the same time metabolic waste products diffuse out of the extracapillary space (ECS) and are washed away into the perfusing culture medium (reservoir bottle (Fig. 1.7) (Shueh et al., 1992).

In conventional methods, cells have to grow on a two-dimensional surface which is different from the physiological microenvironment on which cells normally grow. By contrast, artificial capillary cell culture provides a three-dimensional environment for cell growth (Knazek et al., 1972). In this system there is cell-cell contact, rapid influx of nutrients and outflux of waste material to ECS which provides an environment similar to physiological conditions (Knazek et al., 1990). Figure 1.7 shows the schematic diagram of an artificial capillary system.

Three types of cartridges are used for capillary system: large (LPS), medium and small pore size (SPS) cartridge modules. LPS cartridges allow rapid diffusion across the capillaries of most macromolecules secreted by the cells or present in the medium. By contrast, SPS cartridges do not allow macromolecules such as immunoglobulin to pass the capillary membrane so that immunoglobulins concentrate in the ECS. The yield of

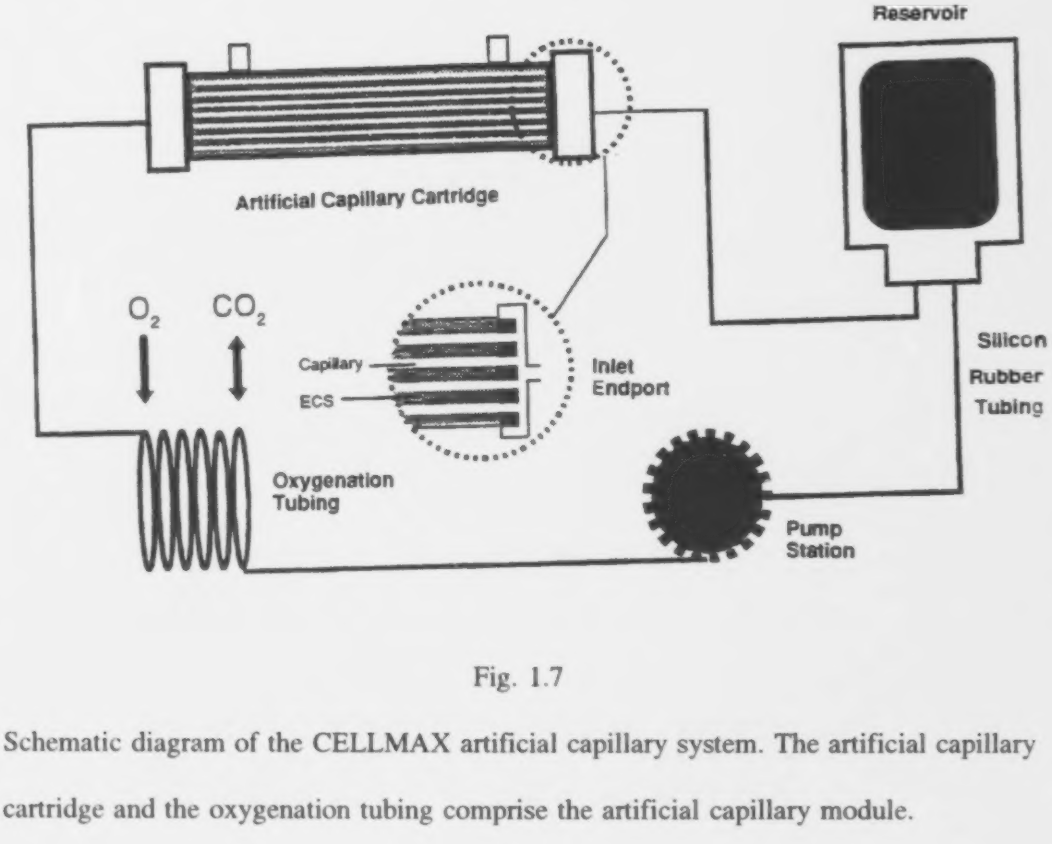

Reservoir
Artificial Capillary Cartridge
O_2
CO_2
Capillary
ECS
Inlet Endport
Silicon Rubber Tubing
Oxygenation Tubing
Pump Station

Fig. 1.7

Schematic diagram of the CELLMAX artificial capillary system. The artificial capillary cartridge and the oxygenation tubing comprise the artificial capillary module.

conventional flask or roller bottle techniques is low (about 20 $\mu g\ mL^{-1}$) so that ascites technique is used for production of large quantities of MAb. With the ascites technique, the yield of Ab is about 1-5 mg mL^{-1}. However, the ascites technique cannot be used when the cell line producing MAb is not syngeneic or it is a heterohybridoma. In such cases, foreign cells act as xenografts and induce immune responses which preclude production of MAb.

To overcome problems associated with conventional methods and the ascites technique, artificial capillary membrane approaches look promising. The pores of the cartridge prevent loss of immunoglobulin so that IgG, IgM, or IgA will accumulate in ECS and attain high concentrations. The yield of artificial capillary system is close to ascites techniques with the harvests of about 15-70 mg Ab per day (Knazek et al, 1990). Usually LPS cartridges give better yields but, because of the dilution factor, the concentration of Ab is lower than with SPS cartridges. Another advantage of artificial capillary systems is that they are more adaptable for using culture medium with low serum levels as it can mimic growth conditions *in vivo* and cell-cell interactions (Heifetz et al., 1989). Key parameters which influence Ab production in artificial capillary systems comprise pore size characteristics of the capillary fibres, frequency of harvesting, serum concentration and CO_2 requirements of the cell line (Andersen & Gruenberg, 1987).

1.5.3. Purification of MAb

Purified Ab are required for a number of techniques, including immunoassays, immunoaffinity, immunological blockade, etc. There is a wide variety of methods used for purifying Ab. The correct choice of purification method will depend on a number of variables, including the use for which the Ab are intended, the species in which it was raised, its class and subclass if it is a MAb and the source that will serve as the starting material for the purification (Pouradier Duteil et al., 1990). Ab from serum or ascites can be purified using conventional methods involving precipitation and column chromatography. However, when purifying tissue culture supernatants, the degree of purity will be lower due to low concentrations of the specific Ab. For tissue culture supernatants, usually protein A, protein G, or anti-immunoglobulin Ab affinity columns are used (Pouradier Duteil et al., 1990).

A new method for Ab purification, termed thiophilic adsorption chromatography, has been described and extensively studied (Porath et al., 1985; Lihme & Heegaard, 1990; Oscarsson et al., 1991). It is a highly selective type of layotropic salt-promoted protein-ligand interaction phenomenon (Porath et al., 1985; Oscarsson et al., 1991). This interaction is called thiophilic as it is distinguished by proteins which recognize a sulfone group in close proximity to a thioether (Porath et al., 1985; Belew et al., 1987). Salts which interact with water molecules such as potassium sulfate and ammonium sulfate enhance binding of proteins to thiophilic supports (Belew et al., 1987). Thiophilic adsorbent (T-Gel™ adsorbent) has a high binding capacity towards immunoglobulin derived from various animal species (Belew et al., 1987).

The neuronal control of airway smooth muscle is complex. In all species, the dominant control of airway smooth muscle tone is exerted via the parasympathetic nervous system. While activation of sympathetic nerves in some species induces relaxation of airway smooth muscle, in other species, including human, although β-adrenoceptors are present on airway smooth muscle, the sympathetic innervation to these receptors is sparse or absent. Both NANC excitatory and inhibitory have been identified in several species. TK release from sensory C-fibres may now be considered as an important factor in pathophysiology of bronchial inflammation and hyperresponsiveness associated with asthma. However, the precise contribution of different nervous system is not yet clear. Thus, TK action on multiple targets in the airways and lungs must be taken into account to understand their possible roles as mediators in airway diseases. In this study, the roles of sensory nerves in bronchoconstriction induced in guinea-pig airways were investigated using both pharmacologic and immunologic approaches. The release of endogenous neuropeptides from sensory nerves was achieved via the use of capsaicin. Immunoblockade is relatively a new approach to investigate the role of biologically-active peptides. Therefore, this study also is a validation of this approach as an experimental tool for probing the physiological roles of TK. To do such experiments, large quantities of Ab were needed. We used NC1/34 cell line, a heterohybridoma which secretes monoclonal anti substance P antibody (α-SP MAb), (Cuello et al., 1979) as a source for production of α-SP MAb.

We used guinea-pigs for our studies for several reasons. They are one of the best characterized animals showing AHR and appear to bear substantial resemblances to asthmatic human subjects (Martin, 1994). They have a rich sensory nervous system in their airways (Lundberg et al., 1987) making them appropriate for determination of the role of sensory nerves which was the main purpose of this study. Furthermore, other investigators have shown that it is possible to induce AHR in guinea-pig by using ovalbumin (OA) (Pretolani et al., 1989; Watson et al., 1990).

It is apparent from the review above that the role of afferent nerves, and the mediators that their sensory endings contain, in the pathogenesisis of lung diseases such as asthma is only partly defined. Interestingly, it has been reported that active immunization of guinea-pigs against SP prevents the development of airway hyperresponsiveness in a guinea-pig model of asthma (Ladenius et al., 1991). If active immunization is effective, then passive immunization with an exogenous antibody should be effective in preventing the development of airways hyperresponsiveness in this model. Carefully characterized MAb against SP appeared to have potential not only for passive immunization but also as tools for identifying the role of mediators released by agents, such as capsaicin, that selectively stimulate sensory C-fiber endings. My literature review suggested that the use of MAb in experiments *in vivo* was relatively unexplored. Accordingly, I elected to prepare and characterize a MAb against SP and to attempt to use it as a pharmacological tool. Lastly, in view of the controversy that exists about the value of β-agonists in the treatment of asthma, I used a guinea-pig model of asthma to determine whether adminstration of albuterol for 6 d via osmotic pumps altered airways' responsiveness to histamine and LTC_4.

OBJECTIVES:

The main objective of these studies was to characterize the involvement of sensory C-fibres in inducing bronchoconstriction in guinea-pigs. We used both pharmacologic (selective NKR agonists and antagonists) and immunologic approaches (MAb against SP).

Goals:

1) To establish a reliable method for assessing airways' responsiveness using bronchospastic agents (iv) and vagal stimulation
2) To prepare sufficient quantities of monoclonal α-SP MAb for *in vitro* and *in vivo* experiments
3) To characterize the antigen binding site(s) of the α-SP MAb
4) To determine whether passive immunization with α-SP MAb altered pulmonary responses to exogenously applied and endogenously released SP and NKA
5) To characterize capsaicin-induced bronchospasm in anesthetized guinea-pigs using selective NKR agonists and antagonists and hCGRP and a CGRP antagonist ($CGRP^{8-37}$).
6) To determine whether sustained administration of rac-albuterol via an implanted osmotic minipump enhanced the increased airways' responsiveness to histamine and LTC_4 seen in naive guinea-pigs and guinea-pigs that had been passively immunized with anti-ovalbumin antibody-containing serum (α-OA Ab) and challenged by inhalation of OA (2%)-containing aerosols.

CHAPTER 2

DIFFERENTIATION OF THE EFFECTS OF BRONCHOSPASTIC AGONISTS ON THE CONDUCTING AND DISTENSIBLE AIRWAYS OF ANESTHETIZED, PARALYSED GUINEA-PIGS USING COMPUTERIZED MEASUREMENTS OF PULMONARY FLOW RESISTANCE (R_L) AND DYNAMIC PULMONARY ELASTANCE (E_L)

2.1. INTRODUCTION

Measurement of the bronchospasm induced by injected or inhaled agonists is used widely to assess airways' responsiveness. Although the theoretical basis for differentiating between effects on the conducting and distensible airways was developed in the early years of the 20th century (see reviews, Brown et al., 1991; Mitzner et al., 1992; Freezer et al., 1993), initially it was not widely applied to the measurement of the effects of drugs and other agents on the airways of small animals such as guinea-pigs. Investigators elected to measure effects on the pulmonary system as a whole and did not attempt to differentiate between effects involving changes in flow or elastance (Mitzner et al., 1992). In 1943, Konzett and Rossler devised a method for monitoring the effects of drugs on the airways of artificially ventilated, anesthetized guinea-pigs. Animals were over ventilated at constant volume to a preset maximal pressure and the excess amount of air was measured via a tambour attached to a lever. The increase in the excursion of the lever was proportional to the degree of "bronchospasm". Their method, or modifications of it, is still in use today in some laboratories. However, many investigators now prefer to measure intratracheal pressure in anesthetized animals, artificially respired at a constant physiologic volume to assess bronchospasm - bronchospasm is taken as being directly proportional to the increase in intratracheal pressure observed. The method is easy to establish and yields reproducible responses to bronchospastic agonists injected iv and to vagal stimulation. However, neither of the above methods can differentiate between effects that involve changes in flow resistance that affect mainly conducting airways, e.g., the bronchi and bronchioles, and effects that involve changes in elastance that affect mainly the distensible portions of the lungs, e.g., the alveolar ducts and alveoli (Colebatch et al., 1966; Colebatch & Engel, 1974; Smith et al., 1984; Nagase et al., 1992). Physiologic studies have shown that the autonomic innervation of the lung is confined to the conducting airways. Also, the distribution of receptors for many ligands differs among parts of the lung. Thus, by measuring only changes in intratracheal pressure, much information about any differential effects of nerve stimulation and bronchospastic agonists on the conducting and distensible sections of the lung is lost. Several groups, e.g., Cole et al., 1993, have described computerized methods of determining changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) in small animals such as guinea-pigs. We describe a computerized system for making breath-by-breath measurements of R_L and E_L in artificially respired, anesthetized, paralyzed guinea-pigs using a commercially available data acquisition program. With this system, we compared the effects of methacholine, histamine, 5HT, SP and NKA, all given iv, and bilateral vagal stimulation on R_L and E_L. We found that the bronchospastic effects of agonists and vagal stimulation on the conducting and distensible airways differ and that the efficacy of the treatments in changing R_L and E_L reflected the distribution of ligand binding sites described by others.

2.2. METHODS AND MATERIALS

2.2.1. Animals

Groups (n = 4 or 8) of SPF-quality, female Hartley-strain guinea-pigs (weight range: 300-500 g) obtained from Charles River Inc., St. Constant, Québec were used.

They were housed in laminar flow units (Bioclean™, Hazleton) on grids over trays of rock salt and were fed guinea-pig chow supplemented with apples. Water was allowed *ad lib*.

2.2.2. Methods

2.2.2.1. Measurements

Guinea-pigs were anesthetized with sodium pentobarbital (40-50 mg kg^{-1}, ip) with additional doses (5 mg kg^{-1}, iv) as required. Their tracheas were cannulated about 1 cm caudal to the larynx (PE240, 3 cm) and artificial respiration was applied (minute volume = 10 mL min^{-1} kg^{-1}, pump speed = 20 strokes min^{-1}) with a rodent ventilator (Ugo Basile, Varese, Italy). A jugular vein was cannulated (PE50, 20 cm, attached to a 23G1 needle, total dead space = 0.06 mL) for giving drugs iv. All animals were given succinylcholine (0.03 mg kg^{-1}, iv) to paralyse them and prevent spontaneous respiratory movements that might interfere with measurements. Both vagi were cut to prevent any reflex responses.

Flow rate was measured via a Fleisch 0000 pneumotachograph (Gould Godart BV, Bilthoven, The Netherlands) with its ports attached across a Validyne MP 45-14-871 differential pressure transducer (Validyne Engineering Corp., Northridge, CA). Intratracheal pressure, which approximates transpleural pressure, was measured via one port of a Validyne MP 45-28-871 differential pressure transducer with the other port open to atmosphere. Excitation for the transducers was supplied by preampifiers ("Validyne preamplifier," Buxco Electronics Inc.). Signals from the transducers were collected and digitized using a Metrabyte DAS 20 data acquisition board controlled by Viewdac™, Version 2 software (Asyst Software Technologies Inc., Rochester, NY) running under Windows™ on a 386-based PC clone. The Viewdac™ software presents the user with a sequence of panels that provide visual feedback and enable mouse control. The experimental "menu" was divided into four areas: 1. Preview signals - allowed observation of the "raw" flow rate, pressure and gating signals; 2. Calibrate system - provided baseline adjustment and calibration and adjustment of transducers' sensitivity; 3. Do one experiment - measured pressure and flow rate signals during the gated portion of the breath and calculated R_L and E_L by the method of Uhl and Lewis (1978). A subroutine averaged data for the first 5 breaths as a "control" and calculated changes in R_L and E_L as percent of control. Pressure and flow rate signals, and measurements of R_L and E_L breath-by-breath were displayed in a strip chart format in real time. Calibration data, timing information, pressure measurements and values of R_L and E_L in absolute values and as percent control were stored on disk. 4. Print results provided hard copy output of selected data to a printer. The portion of the breath examined was determined by an adjustable gate driven by a digital timing device (Ediss & Biggs, 1991). Signals were collected only during the inspiratory phase of the pump cycle. Also, the timing device operated a solenoid valve that opened the system to atmosphere during the expiratory phase (Fig. 2.1); this prevented back flow through the pneumotachograph. Total dead space of the system (pump inlet, pneumotachograph, pressure transducers, solenoid, connecting tubing and tracheal cannula) was 16.3 mL.

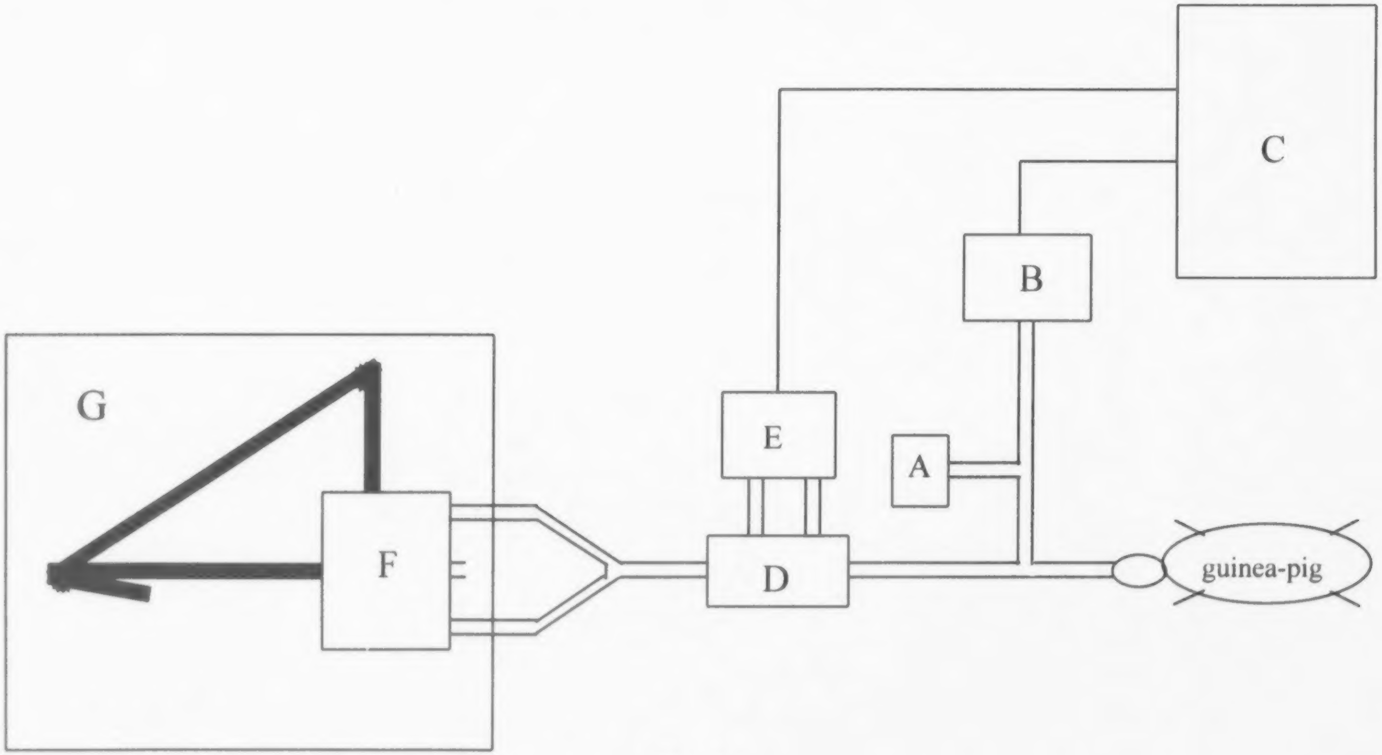


Fig. 2.1

Schematic representation of the system for measuring pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L). A: solenoid; B: pressure transducer; C: computer; D: pneumotachograph; E: differential pressure transducer; F: cylinder; G: ventilator.

2.2.2.2. Evaluation of system for measuring of R_L and E_L

To validate the system of measurement of airway responsiveness, different sets of experiments were carried out. In each experiment changes in resistance or elastance were measured while parameters affecting these values were changed. In one set of experiments, changes in resistance were measured as functions of increases in the length of PE240 tube (length = 5, 15 or 30 cm) attached to the system. Also R_L and E_L were measured at constant minute volume (180 mL min^{-1} kg^{-1}) at various pump speeds (20, 30, 40 or 50 strokes min^{-1}).

2.2.2.3. Nerve stimulation

Both vagi were carefully isolated midcervically and cut. Their peripheral ends were drawn into platinum tunnel electrodes made in this laboratory. The area around the electrodes was flooded with liquid paraffin to prevent current spread to the surrounding tissues. Stimulation was via a constant current stimulator (Biggs & Ediss, 1989). Stimulus parameters were: current = 25-250 mA; frequency = 5 Hz; pulse width = 2 ms. A stimulus counter, built in our laboratory, was used to deliver 8, 16, 24, 32, 40, or 48 stimuli via the electrodes to the nerves. Vagal stimulation was applied in ascending order of number of stimuli. Any changes in R_L and E_L were allowed to recover to baseline before the next stimulation. Each stimulation was repeated once before the next stimulation was applied.

2.2.2.4. Agonists given iv

Guinea-pigs received doses of methacholine, histamine, 5HT, SP, or NKA (all iv) in ascending order of dose until >200% increase in R_L over the baseline was recorded. Each dose was injected in a volume of 0.2 ml and the cannula was flushed with a further 0.2 ml of saline. R_L and E_L were allowed to recover to baseline values between doses. Recovery to baseline was aided by inflating the lungs to twice tidal volme by occluding the outflow port of the solenoid. Each dose was repeated once before giving the next higher dose. Fresh solutions of all drugs were prepared daily in saline.

2.2.3. Materials

Methacholine chloride, 5-hydroxytryptamine creatinine sulfate complex and succinylcholine chloride (Sigma, St. Louis, MO.), histamine dihydrochloride (Fluka AG, Switzerland), substance P and neurokinin A (Peptide Institute Inc., Japan), sodium pentobarbital (Euthanyl™, M.T.C Pharmaceuticals, Markham, ON) and polyethylene tubing (Clay Adams, Becton Dickinson and Company, Parsippany, NJ).

2.2.4. Statistical analyses

Dose- and stimuli-response curves were plotted as mean ± SEM. Data were analyzed using Sigmastat™. Mann-Whitney Rank Sum Tests, Kruskal-Wallis ANOVA and regression analyses of dose-response curves were used to examine differences among responses and to determine linearity and confidence limits. Significance was assumed at the 5% level.

2.3. RESULTS

2.3.1. Measurements of resistance and elastance

At pump speeds ranging from 10-30 strokes min^{-1}, phase shift between the pressure and flow rate signals determined with the Viewdac™ program was minimal (< 5%). At these pump speeds, signals were superimposable during all measurements even at high values of resistance and elastance. At higher pump speeds (> 30 strokes min^{-1}), a phase shift between the two signals became apparent; for this reason, a pump speed of 20 strokes min^{-1} was selected for most experiments.

Values of resistance and elastance of the measurement system itself yielded values that were close to zero (resistance = 0.000-0.006 cm H_2O mL^{-1} min^{-1}; elastance = -0.001-0.004 cm H_2O mL^{-1}). Attaching the 3 cm PE240 tracheal cannula to the system at a pump speed of 20 strokes min^{-1} and pump volume 5 mL gave resistance = 0.048 ± 0.002 and elastance = 0.003 ± 0.001; coefficient of variation was < 5% for these measurements. Attaching animals to the system yielded baseline values of R_L in the range 0.170-0.283 and of E_L in the range 1.500-3.182 (n = 6). Coefficients of variation were < 5% for both R_L and E_L. Variation among measurements was greatly reduced by expanding the lungs with twice tidal volume by occluding the outflow port of the the solenoid for one pump stroke.

Increasing the length of the PE240 tube, attached to the system increased resistance linearly (r^2 = 0.997) (Fig. 2.2). However, changes in pump speed did not alter values of resistance when minute volume was held constant). By contrast, in guinea-pigs, increases in pump speed decreased values of R_L and increased values of E_L (r^2 = 0.954 and 0.943, respectively) (Figs. 2.3-2.4).

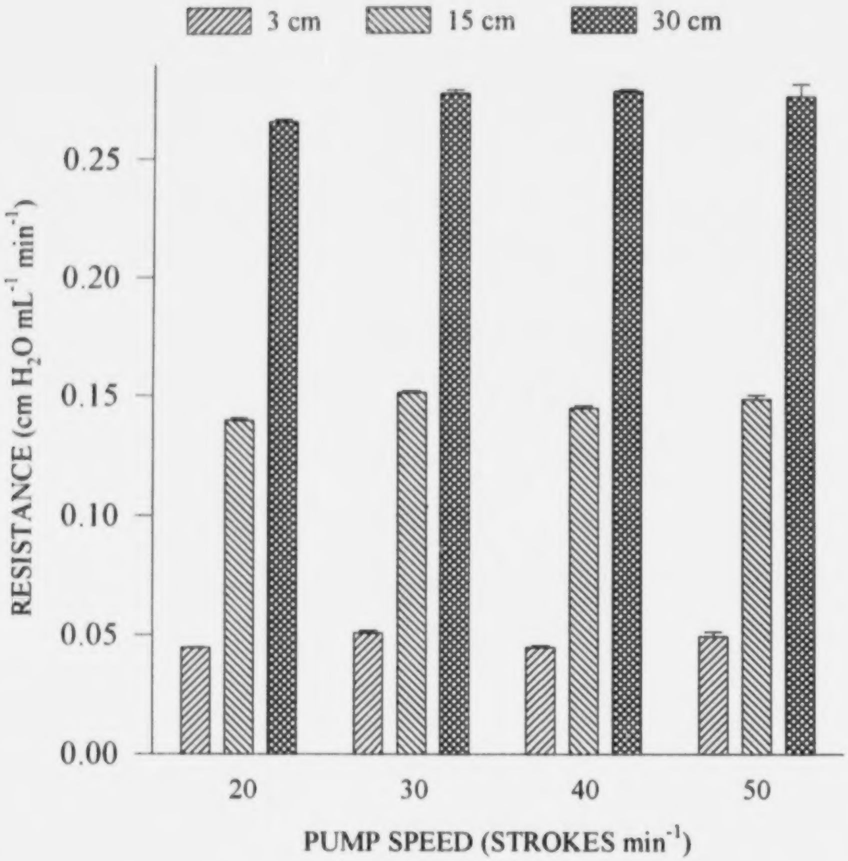


Fig. 2.2

The relationship between pump speed or length of PE240 tube and resistance. Minute volume = 180 mL min^{-1}. Results are mean ± SEM of 10 measurements.

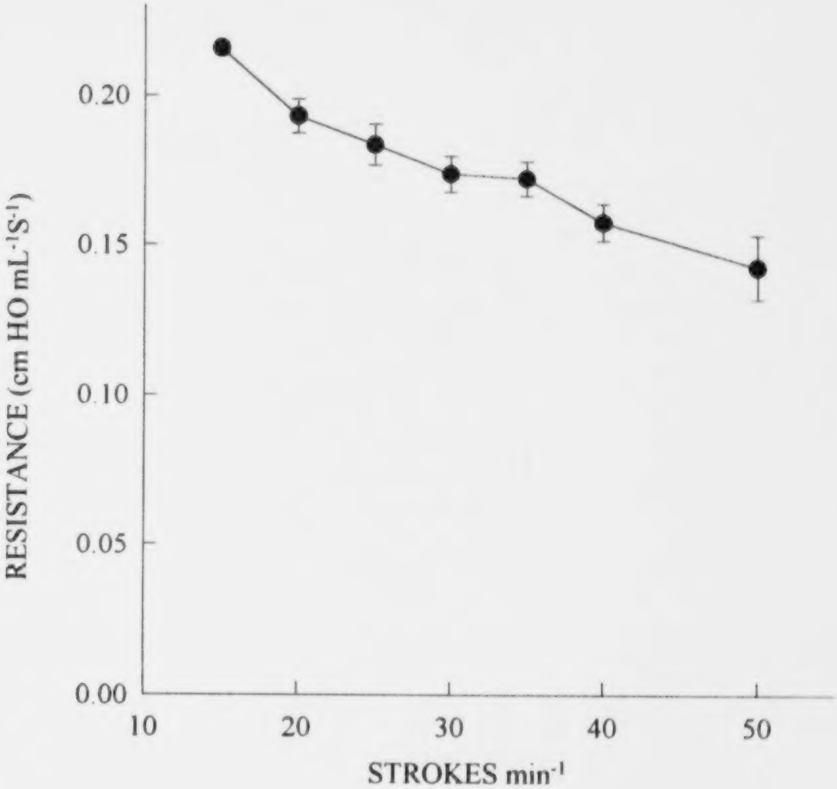


Fig. 2.3

The relationship between pump speed and pulmonary flow resistance in anesthetized guinea-pigs. Minute volume = 180 mL min^{-1} kg^{-1}. Results are mean ± SEM of 3 experiments.

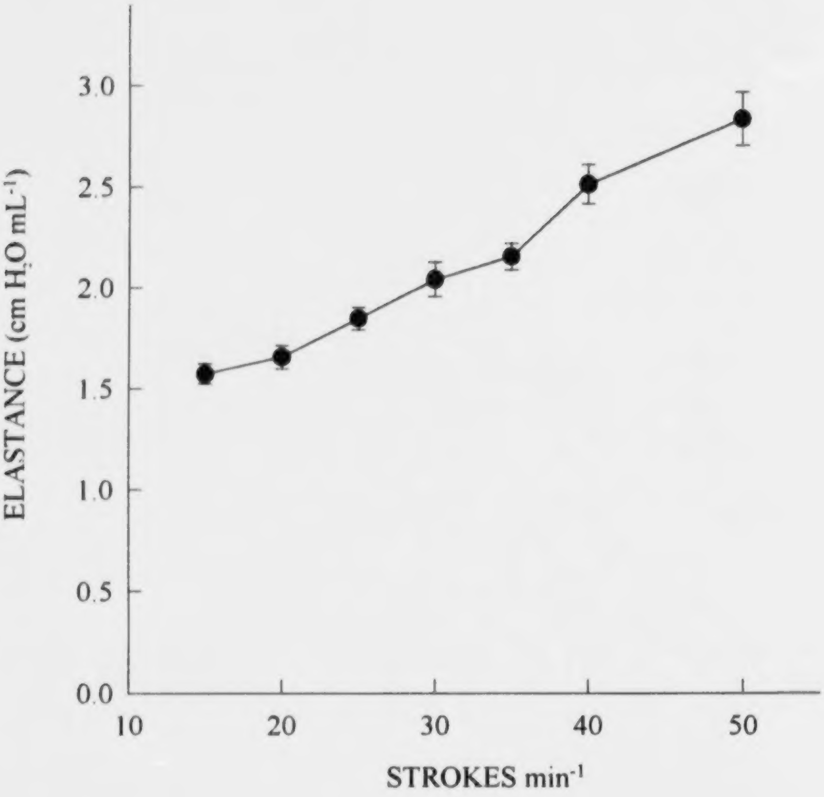


Fig. 2.4

The relationship between pump speed and dynamic pulmonary elastance in anesthetized guinea-pigs. Minute volume = 10 mL min^{-1} kg^{-1}). Results are mean ± SEM of 3 experiments.

2.3.2. Effects of vagal stimulation

Vagal stimulation (8-48 stimuli) at low current intensities (25-100 mA) induced significant changes in R_L, but not in E_L (Fig. 2.5); changes in R_L were abolished by atropine (0.1 mg kg^{-1}, iv), or hexamethonium (2.5 mg kg^{-1}, iv) or mecamylamine (1.0 mg kg^{-1}, iv) (Fig. 2.6). Stimulation with higher currents (> 100 mA) yielded significant increases in both R_L and E_L, however, these changes were not completely blocked by atropine (0.1 mg kg^{-1}, iv) or hexamethonium (2.5 or 5.0 mg kg^{-1}, iv) (see Chow, 1986). At lower current strengths, a plot of log number of stimuli *vs* percent change in R_L from baseline revealed a linear relationship ($r^2 = 0.723$, $p < 0.001$) (Fig. 2.6). There was marked inter-animal variation among the size of the changes in R_L to a single number of stimuli (Fig. 2.7). However, there was no significant intra-animal variation between responses to a set number of stimuli. Figure 2.7 also shows 95% confidence limits for the log number of stimuli-response curve.

2.3.3. Responses to methacholine

Methacholine (0.1-0.4 μg kg^{-1}, iv) induced dose-dependent increases in R_L and E_L (Fig. 2.8). A plot of log dose *vs* percent change in R_L or E_L revealed a linear relationship ($r^2 = 0.61, 0.62$, respectively, $p < 0.05$). Although there were no significant differences within animals, there were significant differences between the changes in R_L and E_L in response to a single dose among animals. Calculation of 95% confidence limits for log dose-response lines indicated that about a 100% increase in change in R_L and a 80% increase in change in E_L from control values were required for significance (Fig. 2.9).

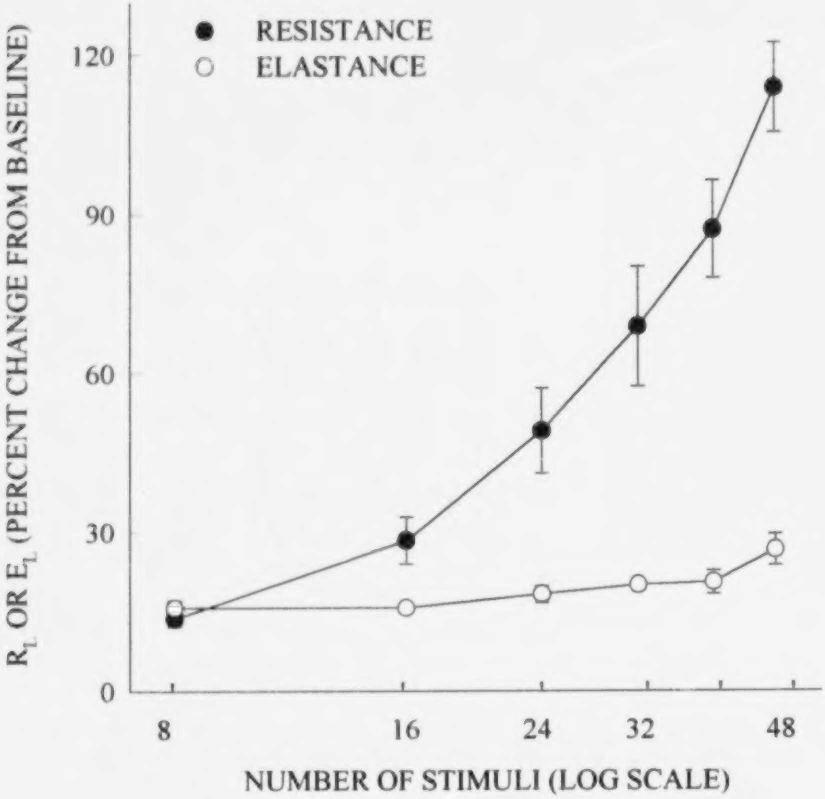


Fig. 2.5

Changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) to varying numbers of stimuli applied to the vagi in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 4 experiments.

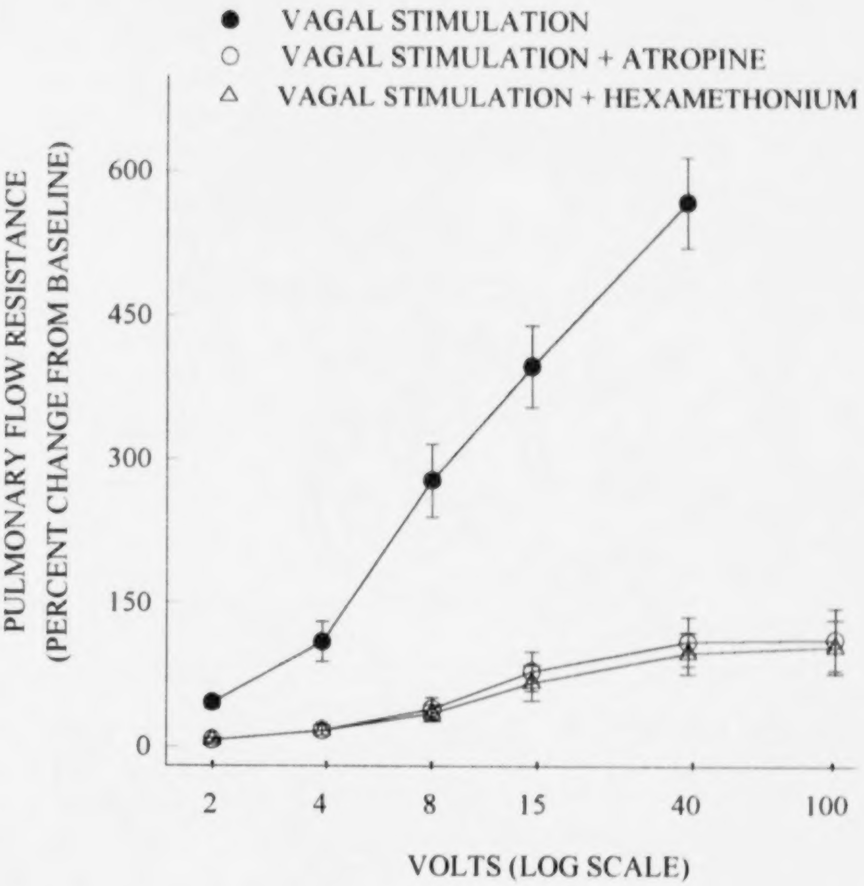


Fig. 2.6

Effects of autonomic blockers on pulmonary flow resistance in response to varying numbers of stimuli applied to the vagi in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 3 experiments.

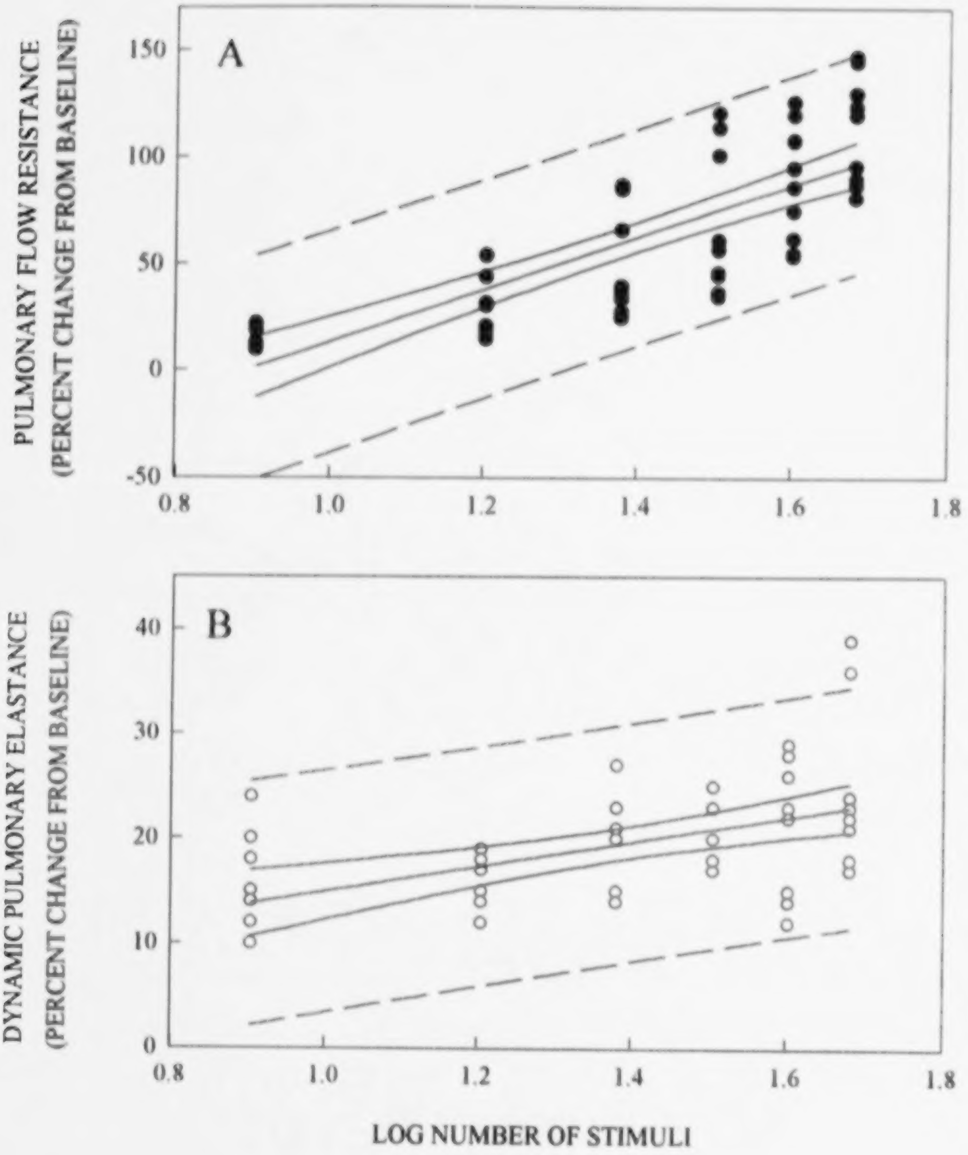


Fig. 2.7

The relationship between percent change in pulmonary flow resistance (A) or dynamic pulmonary elastance (B) and the numbers of stimuli applied to the vagi in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Curved and dotted lines show 95% confidence limits and prediction limits, respectively.

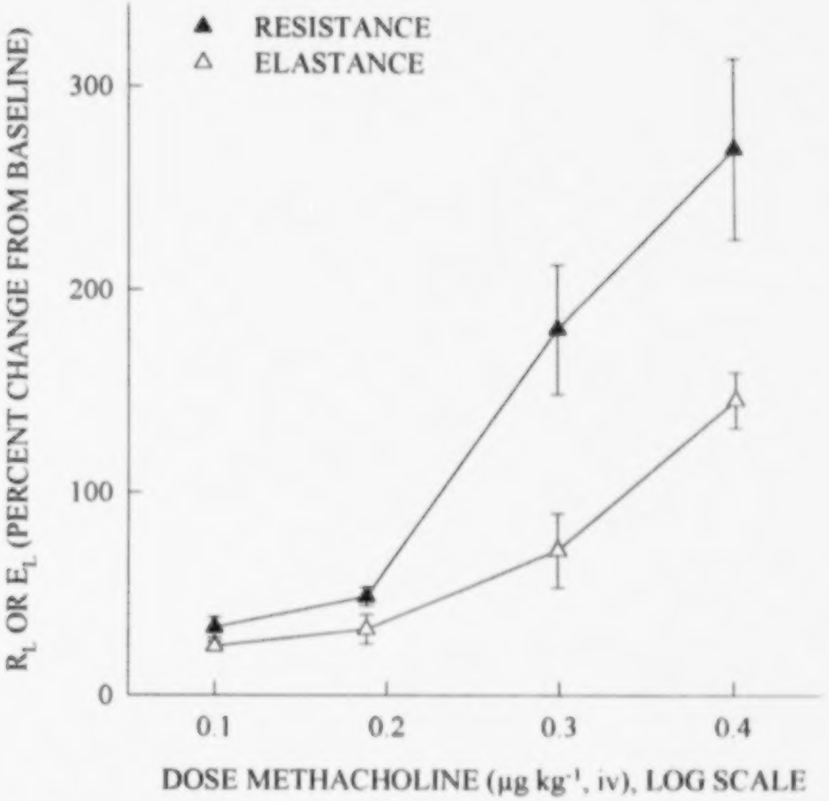


Fig. 2.8

Changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) to various doses of methacholine (iv) in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 4 experiments.

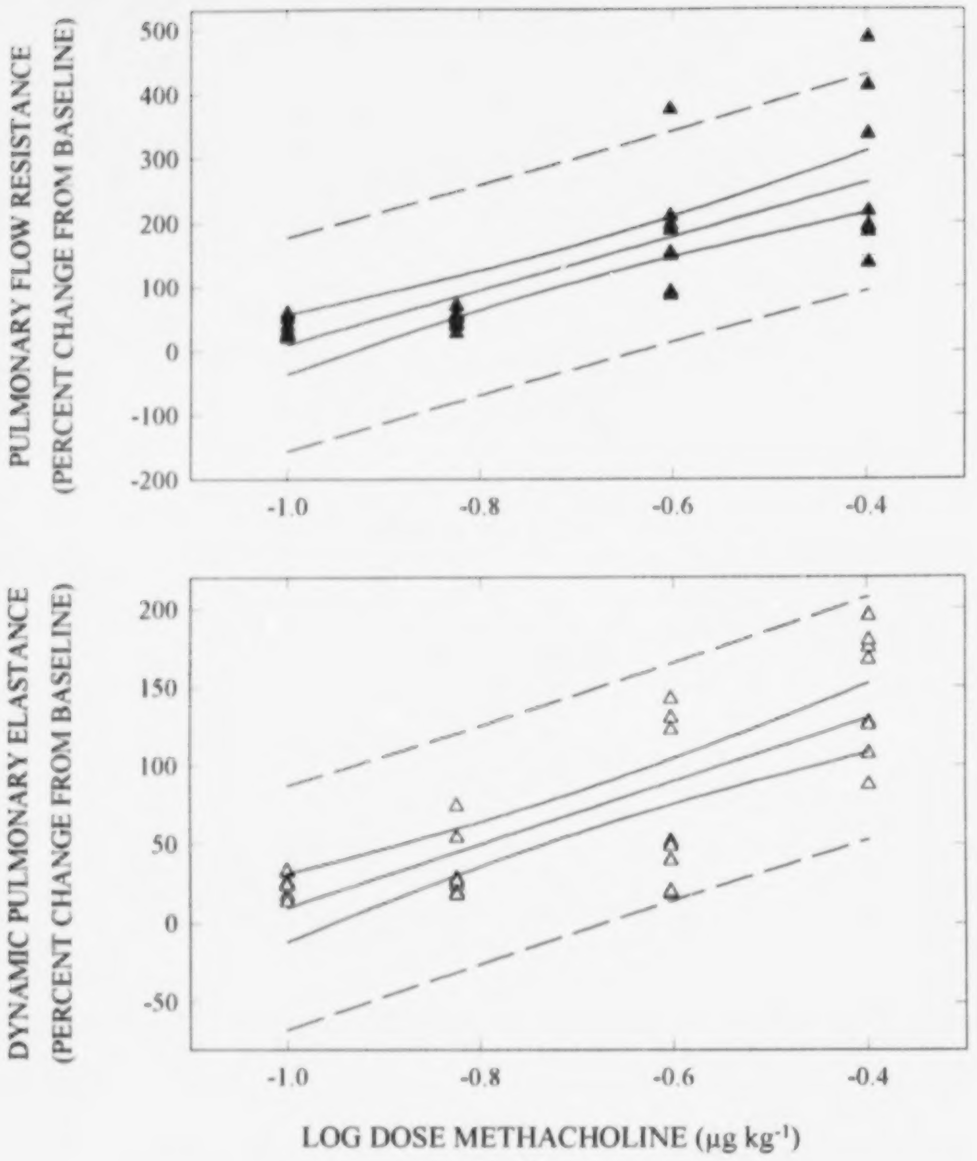


Fig. 2.9

The relationship between percent change in pulmonary flow resistance (A) or dynamic pulmonary elastance (B) and dose of methacholine in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Curved and dotted lines show 95% confidence limits and prediction limits, respectively.

2.3.3. Responses to histamine

Histamine (3.0-10.0 μg kg^{-1}, iv) induced dose-dependent increases in R_L and E_L (Fig. 2.10). A plot of log dose *vs* percent change in R_L or E_L revealed a linear relationship (r^2 = 0.81, 0.63, respectively, $p < 0.05$). As with methacholine, although there were no significant differences within animals, there were significant differences between the changes in R_L and E_L in response to a single dose among animals. Calculation of 95% confidence limits for log dose-response lines indicated that about a 200% increase in changes in R_L and E_L from control values was required for significance (Fig. 2.11).

2.3.4. Responses to 5HT

5HT (5.0-50.0 μg kg^{-1}, iv) induced dose-dependent increases in R_L and E_L (Fig. 2.12). A plot of log dose *vs* percent change in R_L or E_L revealed a linear relationship (r^2 = 0.70, 0.58, respectively, $p < 0.05$, $n = 4$). As with methacholine and histamine, although there were no significant differences within animals, there were significant differences between the changes in R_L and E_L in response to a single dose among animals. Calculation of 95% confidence limits for log dose-response lines indicated that about a 160% increase in changes in R_L and about 60% increase in changes in E_L, from control values were required for significance (Fig. 2.13).

2.3.5. Responses to SP

SP (1.0-4.0 μg kg^{-1}, iv) induced dose-dependent increases in R_L and E_L (Fig. 2.14). A plot of log dose *vs* percent change in R_L or E_L revealed a linear relationship

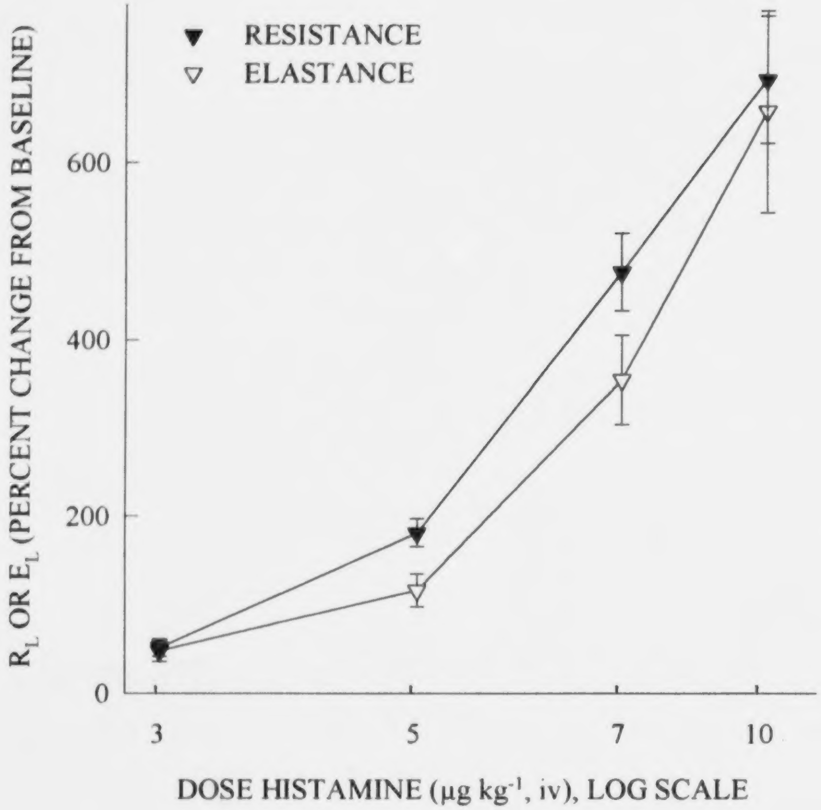


Fig. 2.10

Changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) to various doses of histamine (iv) in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 4 experiments.

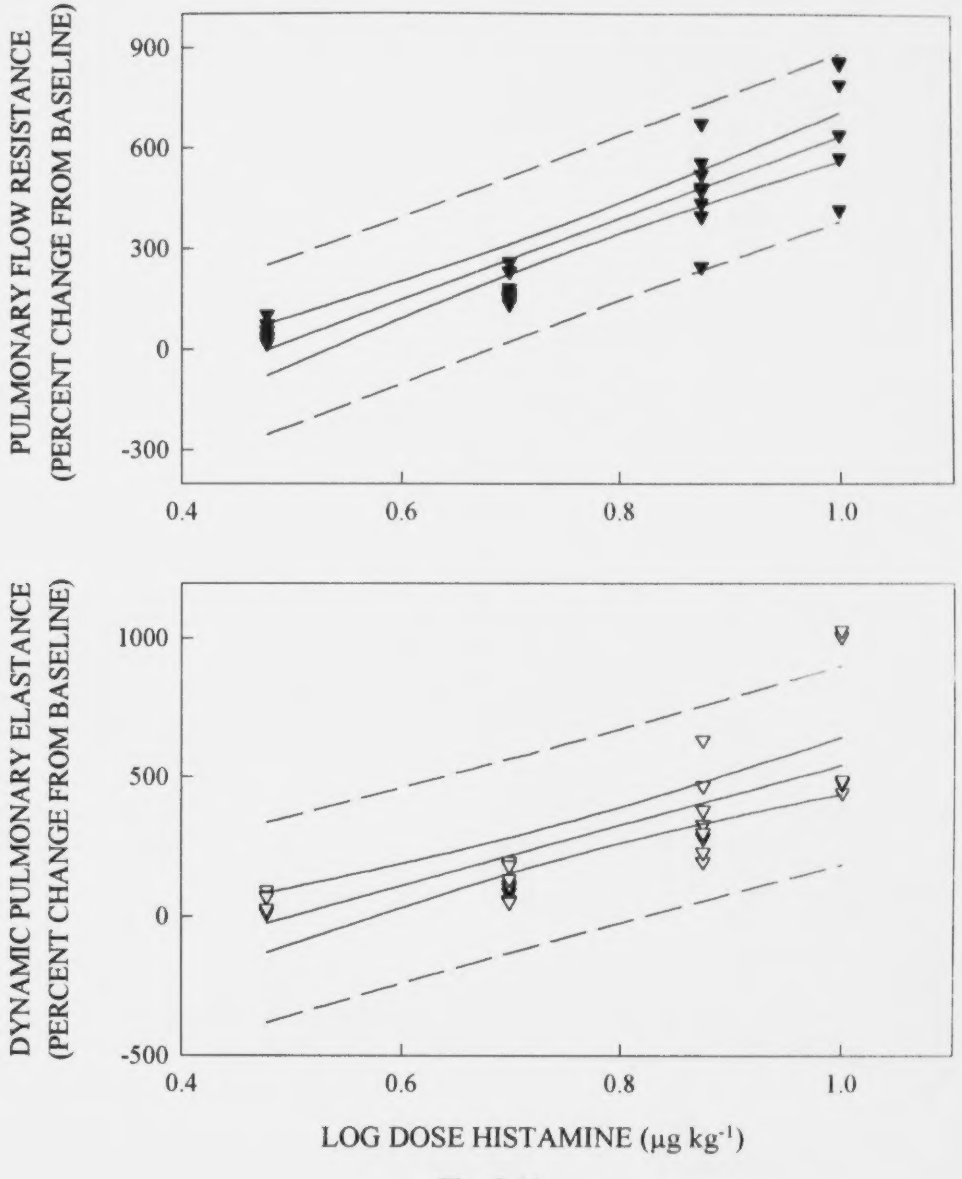


Fig. 2.11

The relationship between percent change in pulmonary flow resistance (A) or dynamic pulmonary elastance (B) and dose of histamine in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Curved and dotted lines show 95% confidence limits and prediction limits, respectively.

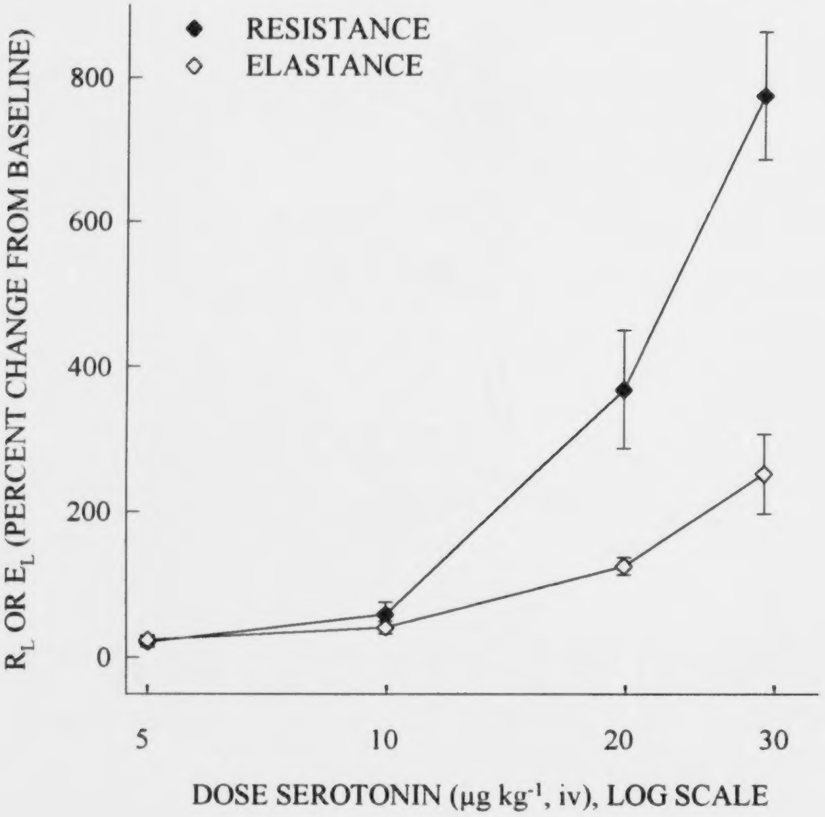


Fig. 2.12

Changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) to various doses of serotonin (iv) in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 4 experiments.

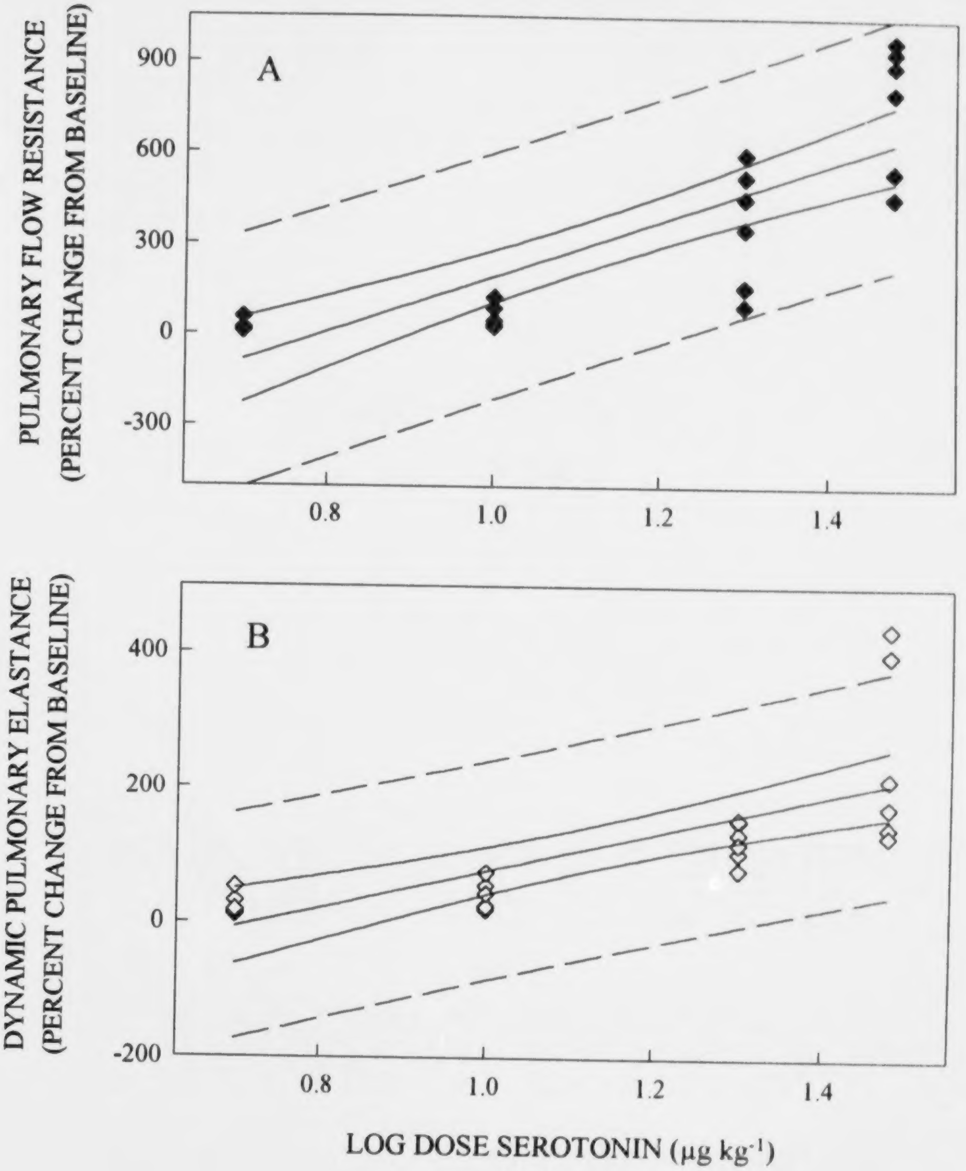


Fig. 2.13

The relationship between percent change in pulmonary flow resistance (A) or dynamic pulmonary elastance (B) and dose of serotonin in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Curved and dotted lines show 95% confidence limits and prediction limits, respectively.

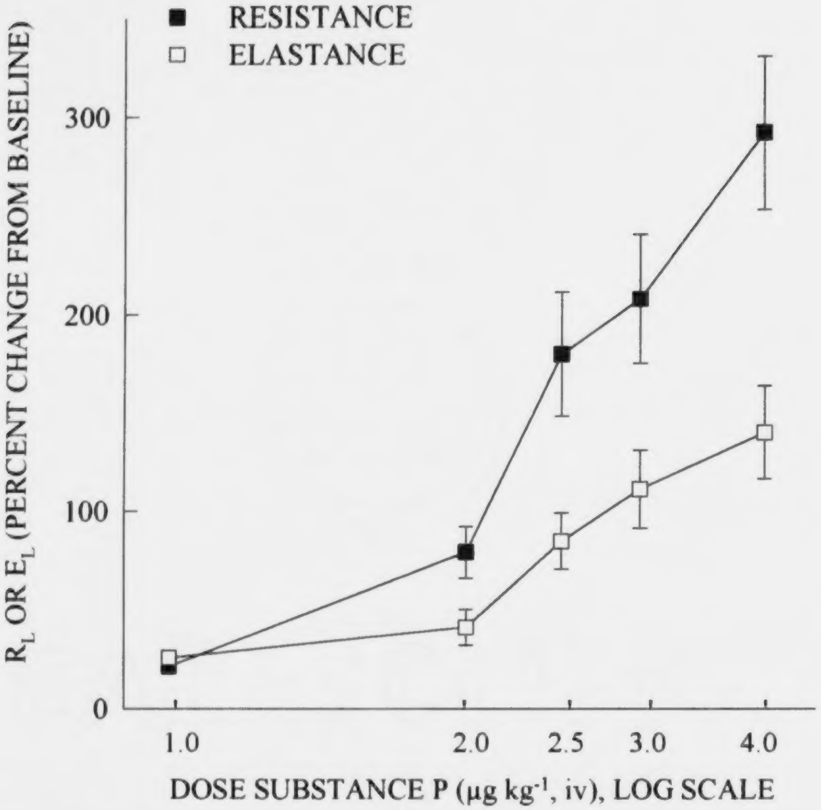


Fig. 2.14

Changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) to various doses of substance P (SP) (iv) in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 6 experiments.

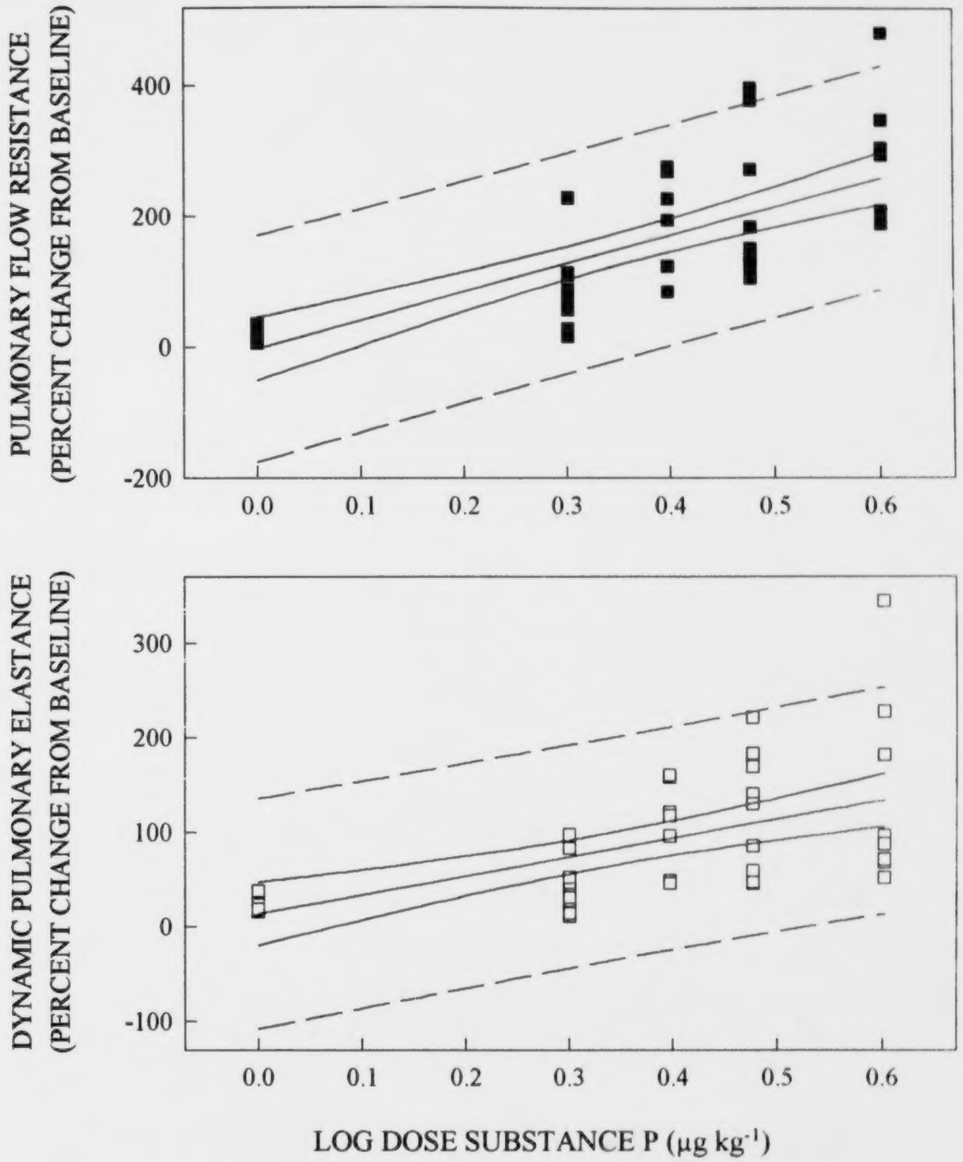


Fig. 2.15

The relationship between percent change in pulmonary flow resistance (A) or dynamic pulmonary elastance (B) and dose of substance P (SP) in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Curved and dotted lines show 95% confidence limits and prediction limits, respectively.

($r^2 = 0.55, 0.54, p < 0.05$). As with methacholine and histamine, although there were no significant differences within animals, there were significant differences between the change in R_L and E_L in response to successive single doses among animals. Calculation of 95% confidence limits for log dose-response lines indicated that about a 100% increase in changes in R_L and about a 70% increase in changes in E_L from control values were required for significance (Fig. 2.15).

2.3.6. Responses to NKA

NKA (0.1-1.0 $\mu g\ kg^{-1}$, iv) induced dose-dependent increases in R_L and E_L (Fig. 2.16). A plot of log dose *vs* percent change in R_L or E_L revealed a linear relationship ($r^2 = 0.72, 0.55$, respectively, $p < 0.05$). As with methacholine and histamine, although there were no significant differences within animals, there were significant differences between the changes in R_L and E_L in response to a single dose among animals. Calculation of 95% confidence limits for log dose-response lines indicated that about a 200% increase in changes in R_L and about 150%increase in changes in E_L from control values were required for significance (Fig. 2.17).

2.4. DISCUSSION

The purpose of these experiments was to examine the natural variation in responsiveness to vagal stimulation and to bronchospastic agonists in guinea-pigs. Vagal stimulation was selected for its known effects on the conducting airways (Cockcroft, 1988). The stimulus parameters used appeared to activate preganglionic parasympathetic

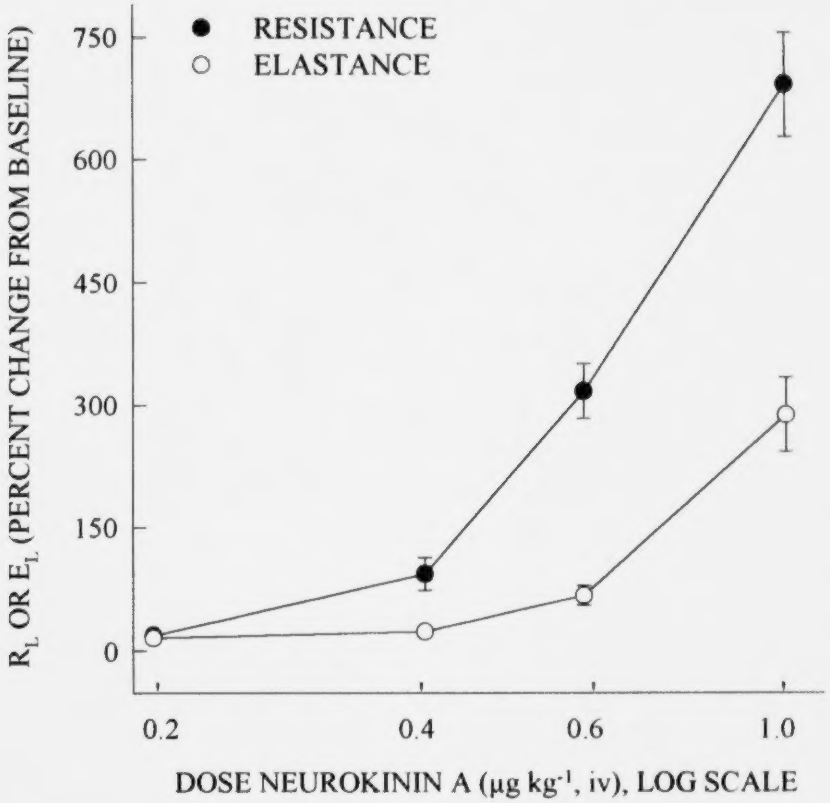


Fig. 2.16

Changes in pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) to various doses of neurokinin A (iv) in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Results as percent change from baseline values are mean ± SEM of 4 experiments.

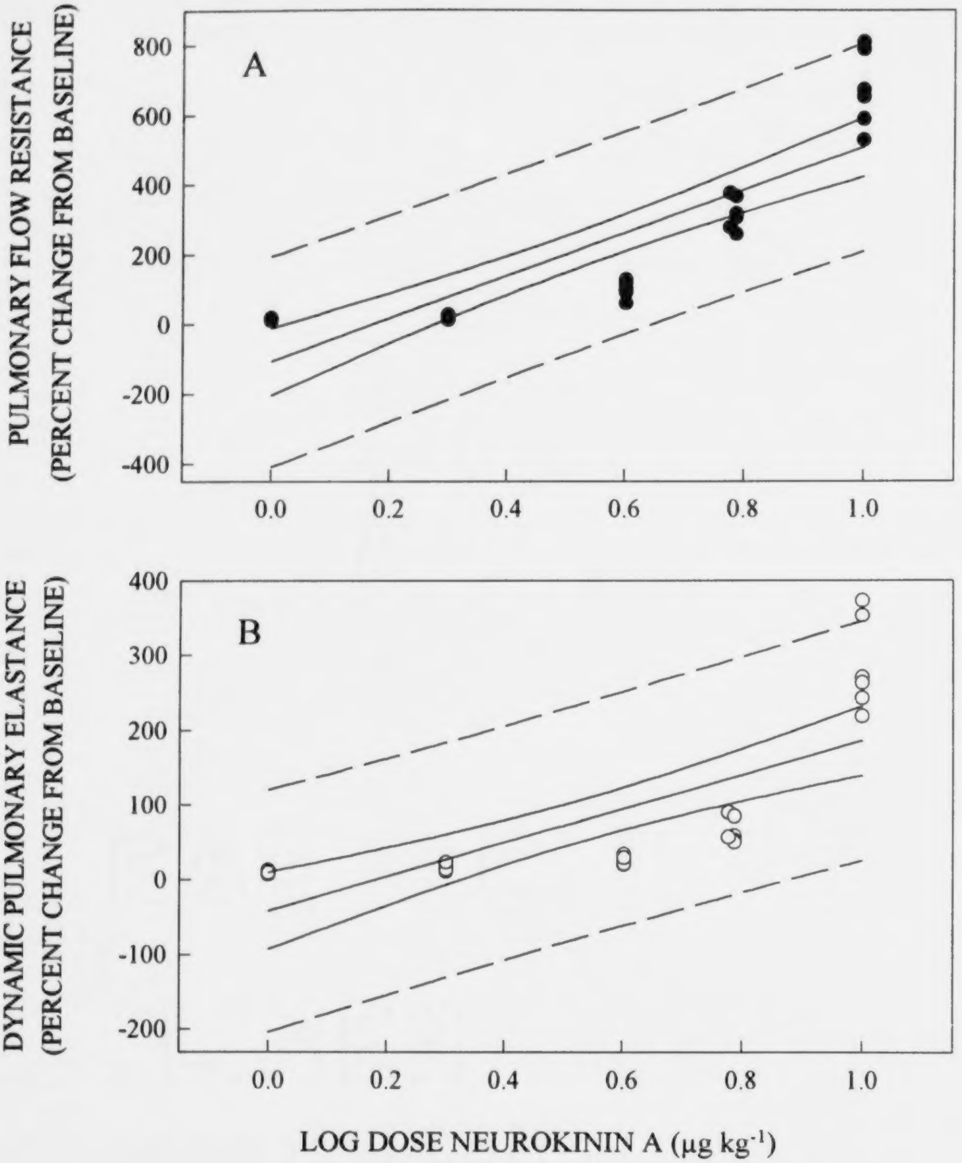


Fig. 2.17

The relationship between percent change in pulmonary flow resistance (A) or dynamic pulmonary elastance (B) and dose of neurokinin A in anesthetized, paralyzed, mechanically ventilated guinea-pigs. Curved and dotted lines show 95% confidence limits and prediction limits, respectively.

nerves selectively as atropine (0.1 mg kg^{-1}, iv) and hexamethonium (50 mg kg^{-1}, iv) eliminated the responses. There was minor evidence of antidromic activation of vagal afferents. Methacholine was selected as an agonist as it is commonly used in bronchoprovocation tests and because of its selective effects on muscarinic receptors in the lung (Hargreave et al., 1981). Histamine was selected because of its involvement in anaphylactic responses. This agonist has the disadvantage that some of its bronchospastic effects may be mediated via autonomic reflexes (Hargreave et al., 1981; Ichinose et al., 1989). However, in these experiments, all guinea-pigs were vagotomized bilaterally to eliminate this potential effect. 5HT was selected as it has been shown that serotoninergic system plays a significant role in upper airway obstruction (Arita et al., 1993). SP and NKA were used as guinea-pigs lungs contain large amounts of these agents and they have been implicated in the development of anaphylaxis and AHR (Lundberg et al., 1983a,b; Pernow, 1983).

To validate the system of measurement of airway responsiveness and to establish the optimal conditions for measuring resistance and elastance, different sets of experiments were carried out. In each experiment changes in resistance or elastance were measured while parameters affecting these values were changed. Applying a constant minute volume and changing pump speed did not alter values of resistance. However, with guinea-pigs attached to the system, decreases in R_L and increases in E_L were noted (Figs. 2.3, 2.4). Although these changes were predicted - the frequency dependence of R_L and E_L is well known - their magnitude was puzzling until we noted that the pressure signals failed to attain baseline between breaths. This suggests that these frequency-dependent changes are a feature of the system. Thus, at higher pump frequencies not all air entering the guinea-pigs was vented via the solenoid's orifice. This results in a measure of positive end expiratory pressure (PEEP) being applied to animals' lungs. Thus distention of the airways would predictably decrease R_L and increase E_L, the changes being proportional to the stroke rate of the pump. To avoid this artefact, all experiments were conducted at a pump stroke rate of 20 per min which eliminated the artefactual changes completely.

The pneumotachograph is a flow-measuring device consisting of a low-resistance element which can be placed in series with subject's airway. The resistance to flow through the pneumotachograph (in the range of flow to be measured) has to be very low compared to that of subject's respiratory system. If flow is laminar the pressure drop across the resistance will be proportional to the flow rate (Poiseuille's law), but if flow becomes turbulent the pressure difference increases disproportionately. Therefore, the limits for linearity must be established for each pneumotachograph (Diamond & Lipscomb, 1970). At high pump speed (strokes $\text{min}^{-1} \geq 50$) the variation of the values of resistance increased probably due to the turbulence induced by rapid air flow.

Measurements of R_L and E_L suggested that vagal stimulation and the various agonists altered these measures of lung responsiveness selectively. Thus, vagal stimulation induced stimuli-dependent changes in R_L. However, methacholine, histamine, 5HT, SP and NKA induced dose-dependent changes in both R_L and E_L. These differences suggest that these agonists affect both the conducting airways and distensible airways, whereas vagal stimulation mainly affects the conducting airways. These findings are in good agreement with those of others (Nadel, 1965; Lundberg et al., 1983b). The findings with vagal stimulation and agonists injected iv indicate that, although responses are reproducible within animals, inter-animal variation in the magnitude of responses to a particular number of stimuli or to a single dose of agonist must be considered when the airways' responsiveness of groups of guinea-pigs is compared. Based on these data, vagal stimulation and/or methacholine, histamine, 5HT, SP and NKA (iv) appear to be suitable tools for comparing airways' responsiveness as effects were reproducible among animals.

CHAPTER 3

PASSIVE IMMUNIZATION WITH AN ANTI-SUBSTANCE P ANTIBODY PREVENTS SUBSTANCE P- AND NEUROKININ A-INDUCED BRONCHOSPASM IN ANESTHETIZED GUINEA-PIGS

A significant portion of this chapter has been published. Jafarian, A., Suresh, M.R., Kreutz, F.T. and Biggs, D.F. 1995. Life Sci. **57**: 143-153.

3.1. INTRODUCTION

SP and NKA belong to a family of closely-related peptides known as the TK. They are widely distributed in the airways and lungs of several species, including humans and guinea-pigs (Maggio, 1988; Guard & Watson, 1991; Maggi et al., 1993). Given parenterally, they induce a variety of responses including contraction of bronchial smooth muscle, mucus secretion, vasodilation, extravasation of plasma proteins and recruitment of inflammatory cells. It has been suggested that release of endogenous SP and NKA from pulmonary afferent C-fibres contributes to bronchial obstruction in asthma (Barnes, 1986a; Lundberg & Saria, 1987; Maggi, 1990). The principal biologic activities of TK reside in their structurally similar carboxyl sequence: ....Phe-X-Gly-Leu-Met-$CONH_2$ (Erspamer, 1981; Gerard et al., 1993). In a guinea-pig model of asthma, active immunization against SP prevented the development of AHR and reduced bronchospastic responses to SP (iv) (Ladenius et al., 1991). We determined whether passive immunization with a monoclonal, IgG_{2a} anti-substance P antibody (α-SP MAb) derived from the rat-mouse heterohybridoma NC1/34 (Suresh et al., 1986) altered pulmonary responses to SP and NKA (iv).

3.2. METHODS AND MATERIALS

3.2.1. Methods

3.2.1.1. Production of α-SP MAb

Two procedures were employed for large quantities of production of α-SP MAb to carry out our experiments.

3.2.1.1.1 Classical method

Frozen NC1/34 cells were thawed and grown in RPMI 1640 medium supplemented with fetal bovine sera (FBS; 10%) and L-glutamine (2 mM). About 1.5 x 10^5 cells ml^{-1} were transferred to RPMI 1640 medium (50 mL) supplemented with 0, 2.5 or 5% FBS and L-glutamine (2 mM) and incubated for 3 d (37° C, 7% CO_2). FBS (2.5%) gave good yields of α-SP MAb. Additional cells were grown in 400 mL flasks or 1 L roller culture bottles. Supernatants (about 10 L) were collected by centrifugation (3000 rpm, 10 min). Cell viability was assessed by trypan blue exclusion.

3.2.1.1.2. Bioreactor (artificial capillary module)

The CELLMAX™ artificial capillary system was used for production of large quantity of α-SP MAb from heterohybridoma NC1/34.

1. System set up: Prior to setting up the system, the reservoir bottle with its cap assembly loosely attached and all other parts of the system, excluding the pump motor, were steam autoclaved at 121°C for 20 min. The artificial capillary module was connected to the reservoir bottle (see Fig. 1.6). Air was removed from extracapillary space (ECS) of the cartridge and replaced with culture medium (serum free media). Then 100 ml of culture medium was added to reservoir bottle and perfused for 2 d to condition the system for cell culture and to ensure sterility of the system. Then, cells precultured for 2 d were inoculated into the system. All operations were performed using aseptic technique in biosafety cabinets.

2. Inoculation: Initially cells were grown in RPMI 1640 medium supplemented with fetal bovine sera (FBS; 10%) and L-glutamine (2 mM). About 1.5 x 10^5 cells ml^{-1} were transferred in RPMI 1640 medium (50 mL) supplemented with 2.5 or 5% FBS and L-glutamine (2 mM) and incubated for 3 d (37°C, 5% CO_2). Finally the cells were transferred into serum free media (BRL). Cells at a concentration of 4 x 10^7 cells ml^{-1} and viability of 90% were inoculated into ECS and incubated at 37° C with 5% CO_2. Then the reservoir bottle was replaced with a 1 L bottle containing fresh culture medium. The pump was set to medium speed for the first two days and then to high speed for the rest of the time.

3. Feeding and harvesting: The culture medium was replaced with fresh pre-warmed medium daily. Daily aliquots were removed for determination of viable cell concentration and Ab production. These steps were done in biosafety cabinet. Starting on day 4, the cells and the Ab secreted were harvested from ECS daily. To minimize the volume of harvested Ab, before collection of the cells and the Ab the perfusion port were closed so that only ECS content was collected. The ECS was flushed vigorously with 30 ml of fresh culture medium to remove additional cells and secreted products. Then it was refilled with fresh culture medium.

3.2.1.2. Purification of α-SP MAb

Separation of α-SP MAb from culture supernatant was attempted with 3 chromatographic methods: protein A-sepharose, protein G-sepharose and thiophilic gel (T-Gel™).

Protein A- and protein G-sepharose - Supernatant was treated with ammonium sulfate (50% saturated solution). The precipitate was collected by centrifugation, resuspended in phosphate buffer (10 mM, pH = 7.4) and dialysed against phosphate-buffered saline (PBS: pH = 7.4; 3 changes over 96 h). A 2 mL protein A-sepharose column was prepared and equilibrated with binding buffer (glycine: 1.5 M; NaCl: 3 M; pH = 8.9). Dialysed material (about 4 mg protein) was added to the column and washed thoroughly with binding buffer; fractions were collected and their UV absorbance monitored. Bound α-SP MAb was eluted with elution buffer (citric acid: 100 mM; pH = 6.0, 5.0, then 4.0). Columns were regenerated with elution buffer (pH = 3.0). The α-SP MAb content of fractions was estimated by ELISA. A similar procedure was used to try and separate α-SP MAb from supernatant using protein G-sepharose.

Thiophilic gel - A 10 mL thiophilic gel (T-Gel™) column was prepared and attached to a UV monitor. It was equilibrated with binding buffer (potassium sulfate: 0.5 M, sodium phosphate: 50 mM, sodium azide: 0.05%). Potassium sulfate was added to untreated supernatant to a final concentration of 0.5 M. 700-800 mL of the mixture was passed through the column and washed through with binding buffer; UV absorbance was monitored and fractions collected. Bound α-SP MAb was eluted with elution buffer (sodium phosphate: 50 mM; sodium azide: 0.05%). Columns were regenerated with guanidine HCl (8 M). The α-SP MAb content of fractions was estimated by ELISA. Fractions containing α-SP MAb were concentrated with Centriprep™ and their α-SP MAb content re-estimated. To purify the α-SP MAb further, fractions (7 mL) containing α-SP MAb were loaded onto a 15-mL DE52 column and eluted with a phosphate buffer gradient (10-100 mM) and fractions collected. Electrophoresis (SDS-PAGE) was used to estimate α-SP MAb purity. The rat hybridoma YP4 which secretes an IgG-type monoclonal antibody against horse radish peroxidase (HRPO) was cultured and purified similarly to provide a control "irrelevant" rat IgG-type antibody for experiments, *in vivo*. Concentrated fractions of both antibodies were dialysed against 0.9% saline (72 h, 3 changes) before use.

Supernatant obtained from the bioreactor was concentrated using Centriprep™ (cut off: 30,000). The concentrated supernatant was dialysed against normal saline 0.9% (72 h, 3 changes). As SDS-PAGE revealed that dialysed supernatant was about 85% pure, concentrated and dialysed supernatant, without any purification, was used for experiments.

3.2.1.3. Preparation of SP-BSA conjugate

SP (15 mg, 1.2 mL) and bovine serum albumin (BSA) (40 mg, 2.0 mL) were dissolved separately in sodium bicarbonate buffer (0.1 M, pH = 9.2) and mixed. Glutaraldehyde (0.5%, 0.6 mL) was added to the mixture and stirred for 2 h at room temperature (22-26°C). Glycine (75 mg) was added to mixture which was stirred for 1 h at room temperature. This mixture was dialysed (3 d, 3 changes) against distilled water. The SP-BSA conjugate was distributed into vials and stored (-20°C) until used.

3.2.1.4. α-SP MAb ELISA method

The relative activities of the purified α-SP MAb were estimated from dilution curves obtained by ELISA. Microtiter plates were coated with constant amount of SP-

BSA conjugate (1:200 dilution) and incubated overnight at 4°C. After washing (3x) with PBS/Tween (0.05%) (automatic plate washer, Molecular Devices, Maxline Model 4845-02), plates were blocked with BSA (1%) in PBS for 1 h at 37°C. After washing (3x), fractions containing α-SP MAb were added and the plates incubated for 1 h at 37°C. After washing (3x), goat anti-rat IgG antibody/HRPO conjugate was added and the plates incubated for 1 h at 37°C. After washing (5x), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) peroxidase substrate was added and the plates were incubated for 15 min at 37°C. Optical density (OD) was measured at 405 nm immediately after the 15-min incubation (Molecular Devices, V max™, Model 04662, SoftMax® Software Version 2.02). Wells containing no α-SP MAb or no SP-BSA conjugate were used as controls. All assays were performed in duplicate.

The relative affinities of the α-SP MAb derived from NC1/34 for SP, NKA and CGRP were estimated using an inhibition assay (Rath et al., 1988). Microtiter plates were coated and blocked as above. Then, in 4 separate groups of experiments employing four dilutions of α-SP MAb (1:5,000; 1:10,000; 1:16,000; 1:32,000), varying concentrations of SP, NKA, or CGRP (0.005-5000 nM; 25 μL) were added to the wells followed immediately by one of the dilutions of α-SP MAb and the plates incubated for 1 h at 37°C. After washing (3x), goat anti-rat IgG antibody/HRPO conjugate was added and the plates incubated for 1 h at 37°C. After washing (5x), ABTS was added and plates were incubated for 15 min at 37°C; OD was measured as before. Changes in OD in the presence of different concentrations of SP, NKA and CGRP were compared with values obtained in the absence of these peptides. Wells containing only SP-BSA and α-SP MAb, only SP-BSA and SP, NKA, or CGRP, and only α-SP MAb and SP, NKA, or CGRP were among the controls used. All experiments were performed in duplicate. The relative affinity of α-SP MAb for SP, NKA and CGRP was estimated by the method of Van Heyningen et al. (1983). Data were fitted to curves using non-linear regression and the concentration of added SP, NKA, or CGRP that reduced OD to 50% of maximum (IC50) determined for each dilution of α-SP MAb.

3.2.1.5. Measurement of airways' responsiveness

Groups (n = 3-5) of SPF-quality, female, Hartley-strain guinea-pigs (weight range: 350-450 g) were used. They were anesthetized with sodium pentobarbital (40-50 mg kg^{-1}, ip, with additional doses, 5 mg kg^{-1}, iv, as required). Their tracheas were cannulated (PE240) and artificial respiration was applied (tidal volume = 9 mL kg^{-1}, pump speed = 20 strokes min^{-1}) with a rodent ventilator (Ugo Basile, Varese, Italy). A jugular vein was cannulated (PE50) for giving drugs iv. Succinylcholine (0.03 mg kg^{-1}, iv) was given to paralyse animals and prevent spontaneous respiratory movements. R_L and E_L were measured continuously, breath-by-breath, using a computerized system. Dose-response (peak response as percent change from baseline values of R_L and E_L) curves to SP (1.0-8.0 μg kg^{-1}), NKA (0.1-1.0 μg kg^{-1}), or methacholine (2.0-10.0 μg kg^{-1}) were established in separate test (α-SP MAb-treated), control (vehicle-treated or "irrelevant" antibody-treated) groups of guinea-pigs. All test animals received approximately 0.055 μM α-SP MAb (iv) 30 min before giving either SP or NKA. Control antibody-treated animals received a similar amount of protein to the test animals.

3.2.2. Materials

RPMI medium 1640, BSA and FBS (Gibco Laboratories, Grand Island, NY USA); ABTS peroxidase substrate (Kirkegaard & Perry Labs., Gaithersburg, MD USA); T-Gel™ Adsorbent (Pierce, Rockford, IL USA); protein A- and protein G-sepharose (Bio-Rad, Hercules, CA USA); guanidine hydrochloride, potassium sulfate, sodium azide and sodium phosphate, (Aldrich Chemical Co., Milwaukee, WI USA); sodium pentobarbital (Euthanyl®, M.T.C Pharmaceuticals, Markham, ON); goat anti-rat IgG antibody/HRPO conjugate, methacholine chloride, succinylcholine chloride (Sigma, St. Louis, MO USA); substance P, neurokinin A and calcitonin gene-related peptide (Peptide Institute Inc., Japan); Centriprep™ (Amicon, Beverly, MA USA).

3.2.3. Statistical analyses

Data from ELISA were analyzed with SoftMax®, Microsoft Excel® and Mathematica® software. Inhibition curves were fitted using non-linear regression and compared via 95% confidence limits. IC50s, with 95% confidence limits, were calculated from the curves. Dose-response curves were plotted as mean ± SEM and analyzed using SigmaStat® software, version 5.01. Kruskal-Wallis ANOVA on ranks was used to compare control and test data. Data at specific concentrations were compared using Student's *t* test and the Mann-Whitney rank sum test. Significance was assumed at the 5% level.

3.3. RESULTS

3.3.1. Production and purification of α-SP MAb

NC1/34 cells grown in media containing 2.5 or 5.0% FBS generated similar amounts of α-SP MAb. In both instances, there was good growth with cell viability about 85%. Cells did not grow well in the absence of FBS. 2.5% FBS was selected for large scale production of α-SP MAb as it was felt that this would simplify purification of the α-SP MAb and economize on FBS. About 10 L of supernatant was collected and stored at 4°C until purified. Anti-HRPO antibody from the rat hybidoma YP4 was prepared similarly.

The artificial capillary system was also used for large scale production of α-SP MAb. NC1/34 cells were originally grown in media containing 5.0% FBS then switched to serum-free media. After transferring to the artificial capillary system there was good growth with cell viability about 85-95%. About 600 mL of supernatant was collected and stored at -20°C. High concentrations of α-SP MAb (about 1 mg mL^{-1}) were obtained. Electrophoresis on SDS-PAGE revealed that the crude supernatant contained about 80% α-SP MAb. To conserve material, it was decided to use the supernatant without further purification.

Protein A- or protein G-sepharose chromatography yielded minimal separation of α-SP MAb from other contaminating proteins (Fig. 3.1A); ELISA showed that similar amounts of α-SP MAb appeared in all fractions collected before and during elution. By contrast, the thiophilic gel column provided excellent separation. ELISA showed minimal amounts of α-SP MAb in fractions collected during binding and large amounts of α-SP

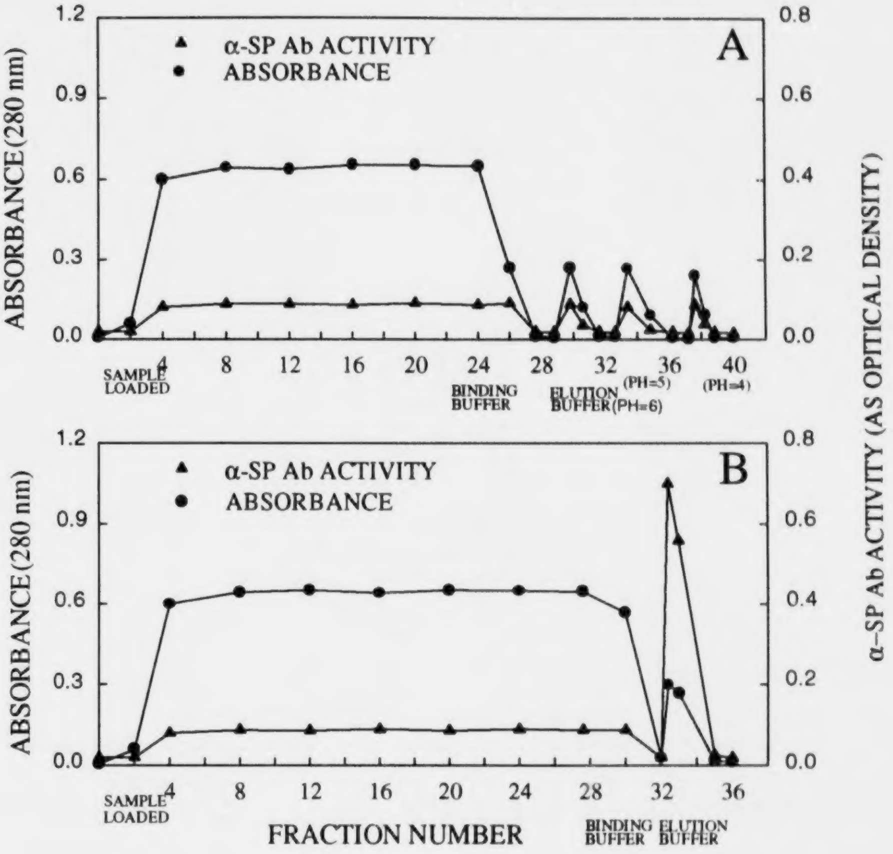


Fig. 3.1

A: Protein A-sepharose chromatography of precipitate from ammonium sulfate-treated supernatant from NC1/34 heterohybridoma; B: Thiophilic affinity chromatography of untreated supernatant from NC1/34 heterohybridoma. Absorbance of fractions at 280 nm and amount of α–SP MAb (as optical density of ELISA) present in each fraction collected are plotted.

MAb in fractions eluted from the column (Fig. 3.1B). Using this procedure, about 20 mg of α-SP MAb was obtained from each litre of supernatant. Electrophoresis on SDS-PAGE revealed that the α-SP MAb was about 50% pure. Attempts to purify the α-SP MAb further using a DE52 column yielded an α-SP MAb that was about 85% pure (SDS-PAGE). However, this purification step was very inefficient and only about 20 - 30% of α-SP MAb activity was recovered. To conserve material, α-SP MAb obtained by thiophilic gel separation was used in all experiments. The control "irrelevant" anti-HRPO antibody derived from the YP4 hybridoma was purified to a similar level of purity using thiophilic affinity chromatography.

3.3.2. Activity and specificity of α-SP MAb

A typical dilution curve for the purified, concentrated α-SP MAb solution was obtained by ELISA using a standardized amount of SP-BSA conjugate to capture the antibody (Fig. 3.2). The relative affinity of the α-SP MAb for SP, NKA or CGRP vs the standardized amount of SP-BSA conjugate coated onto the plates was estimated by adding known amounts of SP, NKA, or CGRP to wells and adding a fixed volume of 4 different dilutions of α-SP MAb. Data are summarized in Fig. 3.3. CGRP did not alter α-SP MAb binding. By contrast, both SP and NKA prevented α-SP MAb binding. At all of the four dilutions of α-SP MAb employed, SP was a better inhibitor of binding than NKA. From the inhibition curves, the ratio of the relative affinities SP/NKA was about 45 (36-52) and 55 (46-65) at α-SP MAb dilutions of 1 in 5000 and 1 in 10000, respectively, but only 6.2 (1.7-10.7) and 3.8 (1.5-6.1) at α-SP MAb dilutions of 1 in 16,000 and 1 in 32,000. 95%

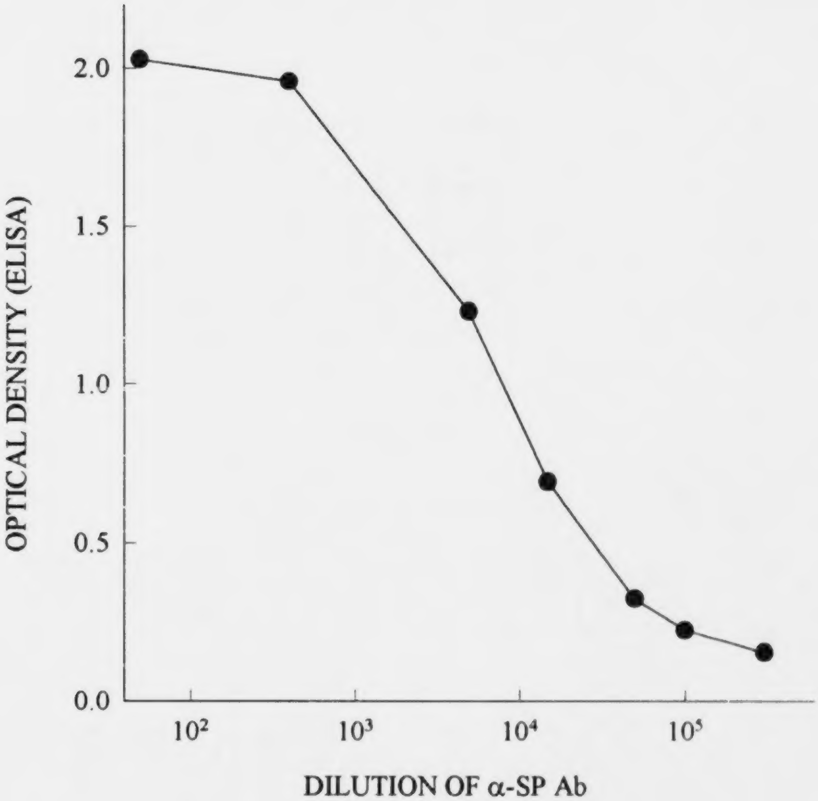


Fig. 3.2

Dilution curve for the anti-substance P antibody (α-SP MAb) obtained using a constant, predetermined amount of SP-BSA conjugate. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

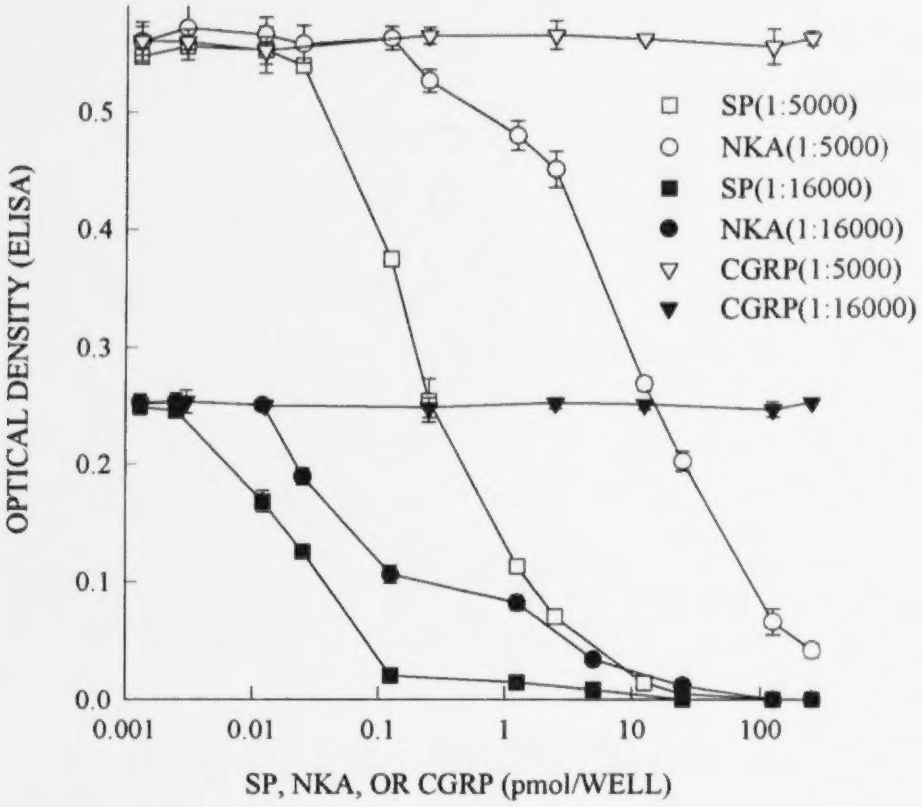


Fig. 3.3

Specificity of anti-substance P antibody (α-SP MAb) evaluated with a competitive binding technique in the presence of a predetermined amount of substance SP-BSA conjugate, 1:5,000 and 1:16,000 dilutions of α-SP MAb (25 μL) and different concentrations of SP, neurokinin A, or calcitonin gene-related peptide (CGRP). The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

confidence limits of the curves showed statistically significant differences between the curves for SP and NKA at α-SP MAb dilutions of 1 in 5,000 and 1 in 10,000, but not at α-SP MAb dilutions of 1 in 16,000 and 1 in 32,000. There was a linear relationship between α-SP MAb dilution and maximal OD in the absence of SP, NKA or CGRP ($r^2 = 0.991$; $p < 0.01$; Fig. 3.4).

3.3.3. Effect of α-SP MAb on bronchospasm induced by SP, NKA, or methacholine

Both SP and NKA induced dose-dependent increases in R_L and E_L in the control groups (Figs. 3.5 and 3.6). On plotting maximal (peak) responses to individual doses, NKA was about 10-12x more potent than SP. In preliminary experiments, SP (5 $\mu g\ mL^{-1}$) in saline was incubated for 2 h at 37°C, alone, or with α-SP MAb in an approximately 1:1 or 1:5 molar ratio. Different volumes of these solutions were used to construct dose-response curves. The findings indicated that the SP:α-SP MAb ratio of 1:5 eliminated SP's bronchospastic effects (Fig. 3.7). This molar ratio was used in all subsequent experiments. Administration of α-SP MAb (iv) in a 5:1 molar ratio to the total cumulative dose of SP (15 μg) did not alter baseline values of R_L and E_L (R_L: before = 0.26 ± 0.03; after = 0.27 ± 0.03; $n = 5$, $p > 0.05$); E_L: before = 2.75 ± 0.43; after = 2.67 ± 0.42; $n = 5$, $p > 0.05$). Baseline values of R_L and E_L in the α-SP MAb-treated, vehicle-treated and "irrelevant" antibody control groups were similar (R_L: test = 0.26 ± 0.03; vehicle control = 0.27 ± 0.03; antibody control = 0.26 ± 0.03; n = 4 and 5, $p > 0.05$; E_L: test = 2.75 ± 0.43; vehicle control = 2.68 ± 0.41; antibody control = 2.63 ± 0.39; n = 3-5, $p > 0.05$). Comparison of the dose-response curves to SP revealed that α-SP MAb

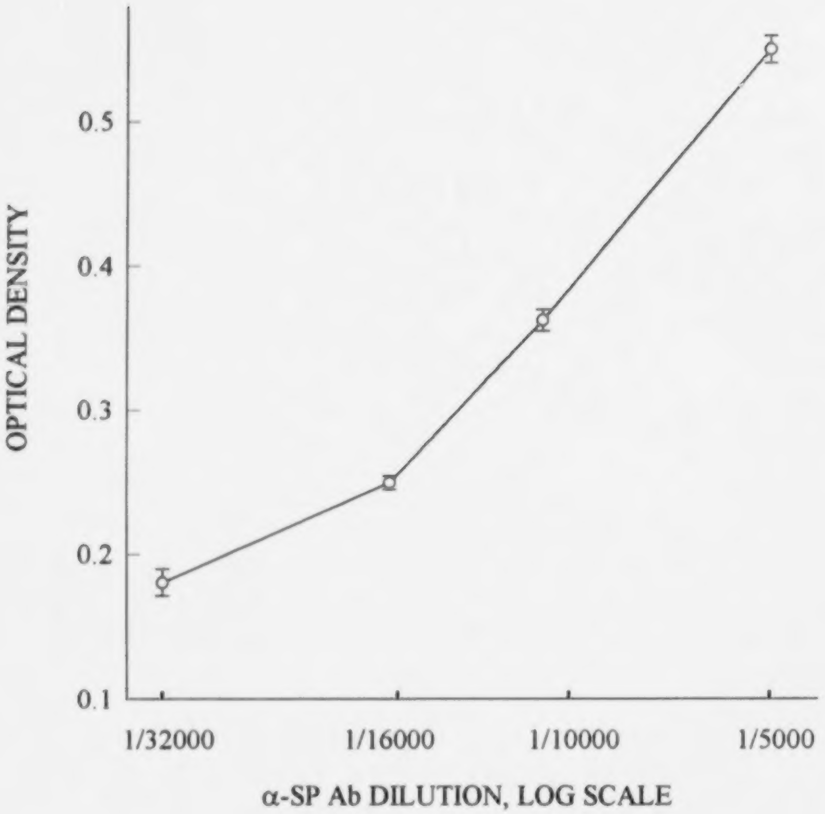


Fig. 3.4

The relationship between optical density in ELISA and amount of anti-substance P antibody. Fixed amount of BSA-SP was coated on the plate.

markedly reduced ($p < 0.05$) changes in R_L and E_L induced by SP (iv). Surprisingly, the same amount of α-SP MAb yielded similar findings with NKA (Figs. 3.5 and 3.6). This amount of α-SP MAb had no effect on changes in R_L and E_L induced by methacholine (Figs. 3.6-3.7). A similar molar ratio of rat IgG-type anti-HRPO MAb used as an "irrelevant" control Ab did not alter responses to SP or NKA (Figs. 3.6 and 3.7).

3.4. DISCUSSION

These findings show that a monoclonal, IgG_{2a}, α-SP MAb derived from the rat/mouse heterohybridoma NC1/34 given intravenously to guinea-pigs prevents increases in R_L and E_L induced by SP or NKA (iv). As an "irrelevant" control, anti-HRPO, IgG-type MAb was without effect, the effects of the α-SP MAb appear to be directed specifically against the TK, SP and NKA. This conclusion is confirmed by the lack of effect of the α-SP MAb against methacholine-induced changes in R_L and E_L. Thus, these experiments using passive immunization confirm those reported previously (Ladenius et al., 1991) which indicated that active immunization of guinea-pigs against SP reduced the bronchospastic response to SP.

NC1/34 cells secrete large amounts of a specific α-SP MAb. This rat/mouse heterohybridoma must be grown in culture as it behaves as a xenograft in mice or rats precluding the use of the ascites technique to produce large amounts of α-SP MAb. Separation and purification of the α-SP MAb from culture media is often difficult and tedious. Neither protein A- nor protein G-sepharose gave a satisfactory absorption and separation of the α-SP MAb even after preliminary separation of protein with ammonium

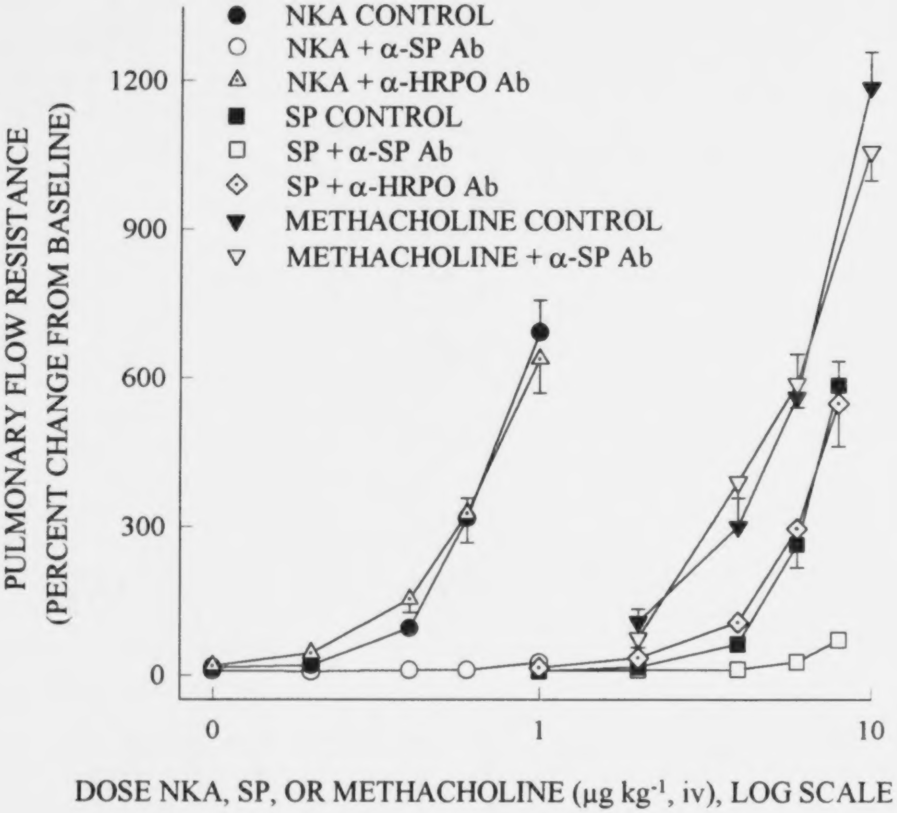


Fig. 3.5

The effect of anti-substance P antibody (α-SP MAb) or α-HRPO MAb on the changes in pulmonary flow resistance in response to various doses of SP, neurokinin A (NKA), or methacholine (MCh) iv. Results, as percent change from baseline values, are mean ± SEM of 4 or 5 experiments.

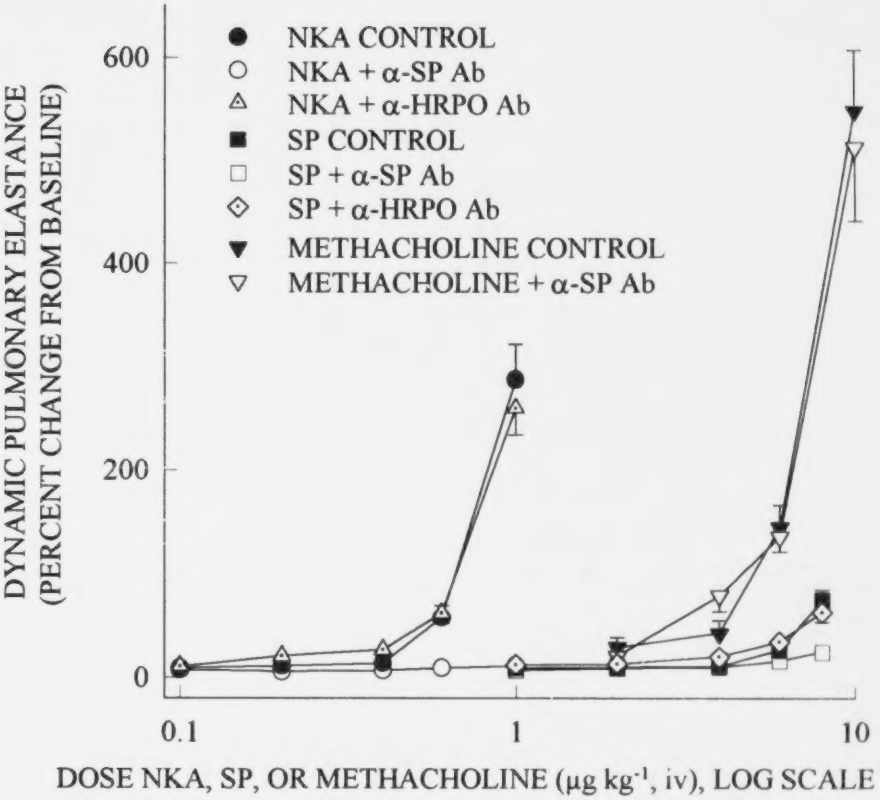


Fig. 3.6

The effect of anti-substance P antibody (α-SP MAb) or α-HRPO MAb on the changes in dynamic pulmonary elastance in response to various doses of SP, neurokinin A (NKA), or methacholine (MCh) iv. Results, as percent change from baseline values, are mean ± SEM of 4 or 5 experiments.

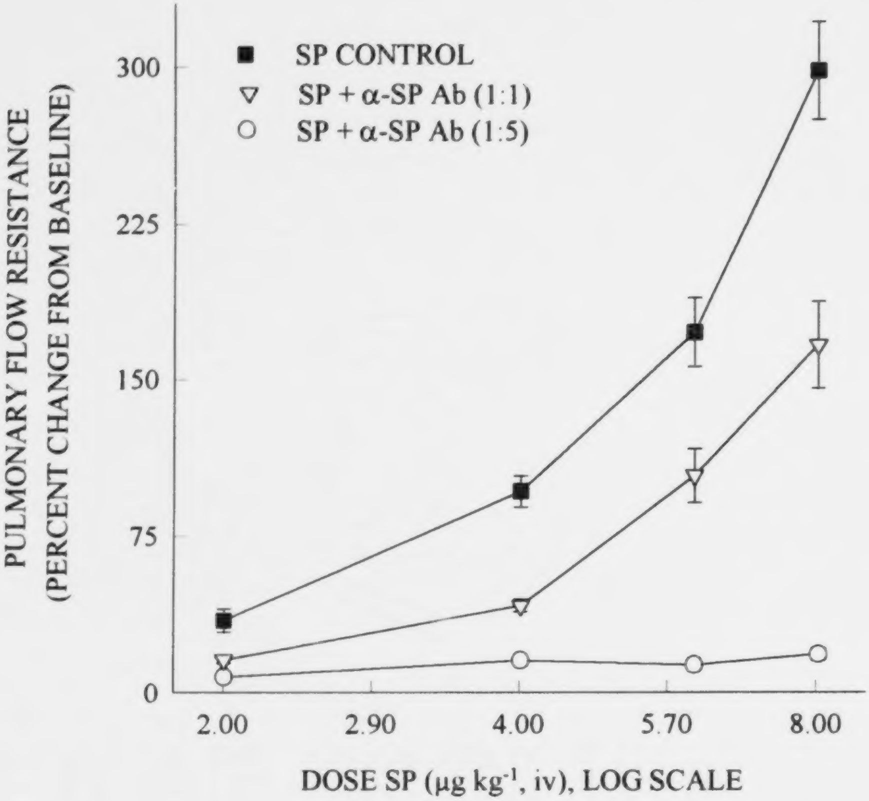


Fig. 3.7

In preliminary experiments, a fixed amount of substance P (SP) in saline was incubated for 2 h at 37°C, alone, or with anti SP antibody (α-SP MAb) in approximately a 1:1 or 1:5 molar ratio. Different volumes of these solutions were used to construct dose-response curves. A SP:α-SP MAb ratio of 1:5 eliminated SP's bronchospastic effects. This molar ratio was used in all subsequent experiments. Results expressed as percent changes from baseline values of pulmonary flow resistance are mean ± SEM of 4 experiments.

sulfate. However, thiophilic gel columns provided excellent absorption of α-SP MAb from raw supernatant; α-SP MAb activity estimated by ELISA was almost completely absent from fractions collected during thiophilic affinity chromatography. α-SP MAb activity was rapidly and completely eluted from the column with low salt buffer. Attempts to purify the α-SP MAb further using a DE52 column were abandoned as, although the purity of the α-SP MAb was improved, yields were very low. α-SP MAb prepared from supernatant by the single step thiophilic gel filtration was about 50% pure by SDS-PAGE and it was decided to use this material, after dialysis against 0.9% saline, without further purification. The efficiency of thiophilic gel purification was confirmed with anti-HRPO MAb obtained from cultures of the rat hybridoma YP4.

The relative affinities of the α-SP MAb for SP, NKA and CGRP were estimated with a competitive binding technique that used a constant, predetermined amount of immobilized SP-BSA conjugate to "capture" 4 different dilutions of α-SP MAb in the presence of different concentrations of SP, NKA or CGRP (Van-Heyningen et al., 1983; Rath et al., 1988). Relative affinities indicated that α-SP MAb had about 50x greater affinity for SP than NKA at α-SP MAb dilutions of 1 in 5,000 and 1 in 10,000. However, at the two lowest dilutions, the ratio of the relative affinities was only about 5. The α-SP MAb had no affinity for CGRP at any of the 4 dilutions of α-SP MAb evaluated. Conjugating SP to BSA and/or immobilizing it on the plate may alter or impede expression of the relevant epitopic determinant of SP. Thus, the SP-conjugate's ability to compete for α-SP MAb probably differs from that of added free SP or NKA. However, although this is not a "true" competitive inhibition assay, as conditions were similar for

SP, NKA and CGRP, it is likely that the values obtained for the relative affinity of the α-SP MAb are representative (Rath et al., 1988). The NC1/34 heterohybridoma was generated by immunizing mice with an antigen prepared by using glutaraldehyde to couple SP via its N-terminal to keyhole limpet hemocyanin. Thus, the α-SP MAb was most probably directed towards an epitopic site on the C-terminal part of the molecule. As TK, SP and NKA share a similar C-terminus - SP: ....Phe-*Phe*-Gly-Leu-Met-$CONH_2$; NKA: ....Phe-*Val*-Gly-Leu-Met-$CONH_2$ - it is not surprising that some cross-reactivity exists.

The preliminary experiments, in which SP was incubated with α-SP MAb before injection of the mixture into guinea-pigs, suggested that the amount of α-SP MAb that must be injected, *in vivo*, to prevent or reduce SP's effects was about a 5:1 molar ratio to the total cumulative dose of SP (Fig. 3.5). Ladenius et al. (1993), who actively immunized guinea-pigs against SP, reported (polyclonal) α-SP Ab titres ranging from 6,000-13,000. "Titres" of α-SP MAb were not determined in the blood of the guinea-pigs used in the current experiments, however, they were anticipated to be at least an order of magnitude less than those reported after active immunization (Ladenius et al., 1993). Nevertheless, the monoclonal α-SP MAb was effective in preventing the bronchospastic effects of both SP and NKA, eliminating or markedly reducing the changes in R_L and E_L induced by these agonists. However, the α-SP MAb was not selective (Figs. 3.6 and 3.7). As NKA was a much more potent bronchospastic agent than SP (Figs. 3.6 and 3.7), the molar ratio of α-SP MAb to the total cumulative dose of NKA was about 40:1, not the 5:1 ratio used for SP. This difference could account for the lack of selectivity. Also, the concentration of the α-SP MAb in serum, *in vivo*, could determine the apparent selectivity of the antibody. If this concentration was sufficiently low, the dilution curve indicated that it could fall on the asymptotic, non-linear portion of the curve. At these dilutions, specificity ratios were about ten-fold lower than at higher concentrations (Fig. 3.2 and 3.3). This would contribute to the apparent lack of selectivity. Lastly, as discussed above, the epitopic site on the antibody may not be as specific/selective, *in vivo*, as has been reported, *in vitro*, by others (Cuello et al., 1979) and in this paper.

An α-SP MAb that prevents the effects of TK such as SP could be a useful tool for determining the role of TK in the pathogenesis of asthma and related lung diseases. The Ab differs from classical pharmacologic antagonists as it would have no effect on NK1, NK2 and NK3 receptors; yet it should prevent, rather than block, most of the pharmacologic effects of exogenous, or endogenously released, TK such as SP and NKA. The apparent lack of specificity, *in vivo*, of the α-SP MAb derived from the rat/mouse heterohybridoma NC1/34 is disappointing as it appeared to be promising, *in vitro*. As suggested above, this could arise from the C-terminal epitopic similarity between the SP and NKA molecules, from the inherent difference in the molar ratios, α-SP MAb:SP or NKA employed, or from dilution factors. Antibodies directed against the respective N-terminals might be more specific and better able to differentiate between the two TK molecules, *in vivo*.

In conclusion, these experiments show that passive immunization of guinea-pigs, acutely, with a monoclonal α-SP MAb prevents the bronchospastic effects of exogenous SP and NKA, *in vivo*, and indicate that this type of passive immunization could be useful for determining the role of TK in guinea-pig models of asthma.

CHAPTER 4

CHARACTERIZATION OF THE MONOCLONAL ANTI-SUBSTANCE P ANTIBODY DERIVED FROM NC1/34

....

A version of this chapter has been submitted to Life Sciences for publication. Jafarian A. and Biggs D.F.

4.1. INTRODUCTION

SP belongs to a family of closely-related peptides known as the TK (Maggio et al., 1985; Guard & Watson, 1991; Maggi, 1993a) whose biologic activities reside on their structurally similar carboxy terminal sequence: ....Phe-X-Gly-Leu-Met-CONH$_2$ (Erspamer, 1981; Gerared et al., 1993). TK, SP and NKA, are widely distributed and active in both the CNS and the periphery (Maggi, 1993). TK's pharmacologic effects are mediated via the interaction of their C-terminus residues with specific NKR (NK1-3) (Maggi, 1990; Frossard & Advenier, 1991; Maggi, 1993b). However, it has been shown that some of the effects of TK such as behavioral effects are mediated by N-terminal of these peptides, where there is considerable variability in amino acid composition (Maggi, 1990; Frossard & Advenier, 1991; Gerhardt et al., 1992; Larson & Sun, 1994). Monoclonal antibodies with high specificity have potential for investigating the roles of naturally occuring peptides. Cuello et al. (1979) developed a rat-mouse heterohybridoma (NC1/34) which produces monoclonal antibody against SP (α-SP MAb) and used it to detect SP in the CNS. They showed that α-SP MAb had about 5% cross reactivity with another related peptide, eleidosin. Some C-terminal fragments of SP were also as well bound as SP in an inhibition radioimmunoassay (Cuello et al., 1979). We reported previously that α-SP MAb had some cross reactivity with NKA (Jafarian et al., 1995). In these experiments, we used inhibition ELISA or dot immunoassay to determine the relative affinities of α-SP MAb for SP and its fragments. Also, we examined SP and SP$^{6-11}$ analogues with sequential substitution of alanine for each of their amino acids to identify the amino acids involved in α-SP MAb binding to SP.

4.2. METHODS AND MATERIALS

4.2.1. Methods

4.2.1.1. Preparation of α-SP MAb

α-SP MAb was prepared from the supernatant of NC1/34 rat-mouse heterohybridoma and purified as described previously (see chapter 3 and Jafarian et al., 1995).

4.2.1.2. Preparation of SP-BSA conjugate

SP (15 mg, 1.2 mL) and bovine serum albumin (BSA) (40 mg, 2.0 mL) were dissolved separately in sodium bicarbonate buffer (0.1 M, pH = 9.2) and mixed. Glutaraldehyde (0.5%, 0.6 mL) was added to the mixture and stirred for 2 h at room temperature (22-26°C). Glycine (75 mg) was added to mixture which was stirred for 1 h at room temperature. This mixture was dialysed (3 d, 3 changes) against distilled water. The SP-BSA conjugate was distributed into vials and stored (-20°C) until used.

4.2.1.3. Measurement of relative affinities by ELISA

The relative affinities of the α-SP MAb for SP and its fragments and selective NKR antagonists were determined by inhibition ELISA (Jafarian et al., 1995). Briefly, microtiter plates (Nunc-Immuno Plate, MaxiSorp™) were coated with constant amount of SP-BSA conjugate and incubated overnight at 4°C. After washing (3x) with PBS/Tween (0.05%) (automatic plate washer, Molecular Devices, Maxline Model 4845-

02), plates were blocked with BSA (1%) in PBS for 1 h at 37°C. Then, varying concentrations of SP, SP fragments, or selective NKR antagonists (0.04-1.00 μM, 25 mL) were added to the wells; a constant amount of α-SP MAb solution was added and the plates incubated (1 h, 37°C). After washing (3x), rabbit anti-rat IgG antibody/HRPO conjugate was added and the plates incubated (1 h, 37°C). After washing (5x), ABTS was added and OD was measured at 405 nm after 15 min (Molecular Devices, V max™, Model 04662, SoftMax® Software Version 2.02). All assays were performed twice in duplicate. Changes in OD in the presence of different concentrations of SP, SP fragments, or selective NKR antagonists were compared with values obtained in the absence of these peptides. Wells containing only SP-BSA and α-SP MAb, only SP-BSA and SP, or SP fragments and only α-SP MAb and SP or SP fragments were among the controls used. The relative affinities of α-SP MAb for SP and SP fragments were estimated by the method of Van Heyningen et al. (1983). Data were fitted to curves using non-linear regression and the concentrations of added SP, SP fragments, or selective NKR antagonists that reduced OD to 50% of maximum (IC50) were estimated.

4.2.1.4. Synthesis of peptides

A SPOTs (solid phase synthesis of peptides) schedule was generated (SPOTs software, version 2). Fmoc-amino acid chlorides were used for the solid phase synthesis. Cellulose membranes with free OH groups were used as the solid phase. All amino acids used were of L configuration. Fmoc-amino acids were dissolved in 1-methyl-2-pyrrolidine (110-180 mg mL^{-1}). A 0.9 μL aliquot of the first amino acid was dispensed on to each of the SPOTs (color change from blue to blue/green, green or yellow) and repeated after 15 min. After 15 min, the trough containing the SPOTs membrane was placed on rocking table and dimethylformamide (DMF) was added to the trough and rocked (2 min). This step was repeated twice. To block any unreacted amino acid, the SPOTs membrane in the trough was treated with acetic anhydride (0.8 mL) in DMF (20 mL) and rocked (15 min). After washing with DMF (3x), excess acetic anhydride was eliminated by adding 20% piperidine in DMF and rocking (5 min). After washing with DMF (3x), 1% bromophenol blue solution in DMF was added to SPOTs membrane and rocked (5 min). After washing with methanol (3x), the SPOTs membrane was wrapped in chromatography paper and dried in a cool air dryer. This procedure was repeated for each cycle in which an amino acid was added, except the last. For the last cycle the amino acid was added as above. After washing (3x) with DMF, 20% piperidine/DMF was added. Then, after washing (3x) with DMF, color was developed with bromophenol blue solution. After washing with DMF (3x), acetic anhydride was added. Then, after washing with DMF (3x) and with methanol (3x), the SPOTs membrane was dried.

After the last cycle, the side chain deprotection was performed by adding a mixture of dicholoromethanol:trifluroacetic acid:triisobutylsilane (1:1:0.05) to the SPOTs membrane in a propylene box and rocking (1 h). After washing with dichloromethane (3x) and methanol (3x), the SPOTs membrane was dried, placed in plastic bag and stored (-20°C).

The sequence of the each peptides synthesized using SPOTs is shown in Table 4.1.

TABLE 4.1

Amino acid sequences of SP^{6-11} and SP analogues synthesized by SPOTs analysis. Double line represents the cellulose membrane.

SP^{6-11}					NH_2	Gln	Phe	Phe	Gly	Leu	Met	COOH
1					NH_2	Gln	Phe	Phe	Gly	Leu	***Ala***	COOH
2					NH_2	Gln	Phe	Phe	Gly	***Ala***	Met	COOH
3					NH_2	Gln	Phe	Phe	***Ala***	Leu	Met	COOH
4					NH_2	Gln	Phe	***Ala***	Gly	Leu	Met	COOH
5					NH_2	Gln	***Ala***	Phe	Gly	Leu	Met	COOH
6					NH_2	***Ala***	Phe	Phe	Gly	Leu	Met	COOH
7	NH_2	Gln	Phe	Phe	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
8	NH_2	Gln	Phe	Phe	Gly	Leu	***Ala***	**Arg**	**Arg**	**Arg**	**Arg**	COOH
9	NH_2	Gln	Phe	Phe	Gly	***Ala***	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
10	NH_2	Gln	Phe	Phe	***Ala***	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
11	NH_2	Gln	Phe	***Ala***	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
12	NH_2	Gln	***Ala***	Phe	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
13	NH_2	***Ala***	Phe	Phe	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
SP	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	NH_2
14	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
15	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	***Ala***	COOH
16	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	***Ala***	Arg	COOH
17	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	***Ala***	Pro	Arg	COOH
18	Met	Leu	Gly	Phe	Phe	Gln	Gln	***Ala***	Lys	Pro	Arg	COOH
19	Met	Leu	Gly	Phe	Phe	Gln	***Ala***	Pro	Lys	Pro	Arg	COOH
20	Met	Leu	Gly	Phe	Phe	***Ala***	Gln	Pro	Lys	Pro	Arg	COOH
21	Met	Leu	Gly	Phe	***Ala***	Gln	Gln	Pro	Lys	Pro	Arg	COOH
22	Met	Leu	Gly	***Ala***	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
23	Met	Leu	***Ala***	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
24	Met	***Ala***	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
25	***Ala***	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH

4.2.1.5. Direct ELISA on SPOTs membrane (dot immunoassay)

All steps were carried out at room temperature. The SPOTs membrane was allowed to warm to room temperature, rinsed with methanol and dried. After washing (3x) with PBS for 10 min, blocking buffer (BSA 3% in PBS) was added to the SPOTs membrane and it was rocked overnight. After washing (3x) with PBS, α-SP MAb or polyclonal anti-SP Ab was added to the SPOTs membrane and it was rocked for 4 h. After washing (3x) with PBS, second antibody (goat anti-rat IgG/HRPO conjugate or goat anti-rabbit IgG/HRPO conjugate) was added to the SPOTs membrane and it was rocked for 2 h. After washing (3x) with PBS, substrate (TM-Blotting™ or 4-chloro-1-naphthol) was added and the membrane rocked (10-60 min) to distinguish between positive and negative SPOTs.

4.2.1.6. Peptide regeneration

After immunoassay, the SPOTs membrane was washed (3x, 10 min) with MilliQ water, then with DMF (3x, 10 min). The membrane was washed (3 x, 10 min) with regeneration buffer A (urea 48% and sodium dodecyl sulphate 1% in MilliQ water) and then (3x, 10 min) with regeneration buffer B (MilliQ water: ethanol: acetic acid, 4:5:1). Finally, it was washed (3x, 10 min) with methanol, before drying and storage in a plastic bag at -20°C.

4.2.2. Materials

ABTS peroxidase substrate (Kirkegaard & Perry Labs., Gaithersburg, MD USA); substance P (Peptide Institute Inc., Japan); goat anti-rabbit IgG antibody/HRPO conjugate, rabbit anti-rat IgG antibody/HRPO conjugate, N,N-dimethlformamide, 1-methyl 2-pyrrolidine, 4-chloro-1-naphthol, SP^{9-11}, SP^{8-11}, SP^{7-11}, SP^{6-11}, SP^{5-11}, SP^{4-11}, SP^{3-11}, SP^{2-11}, SP^{1-4}, SP^{1-7} and SP^{1-9} (Sigma, St. Louis, MO USA); rabbit anti-substance P antibody (ICN Biochemical Inc., Costa Mesa, CA, USA); ethyl alcohol and methyl alcohol (Mallinckrodt Specialty Chemicals Canada Inc., Mississauga, ON Canada); acetic acid and acetic anhydride (Anhaler, BDH Inc. Toronto, ON Canada); dichloromethane, piperidine and methylene chloride (Fisher Scientific Ltd, Nepean, ON Canada); bromophenol blue, trifluoroacetic acid and triisobutylsilane (Aldrich Chemical Company, Milwaukee, WI USA); Fmoc-amino acids, methionine, Phe-Phe, Phe-Gly, Gly-Leu and Leu-Met (Bachem Bioscience Inc., Philadelphia, PA USA); sodium dodecyl sulphate and urea (Gibco BRL, Gaithersburg, MD USA); 3,3',5,5'-tetramethyl benzidin (TMB-Blotting™, Pierce, Rockford, Illinois, USA). CP 96,345 was a gift of Pfizer Inc., Groton, CO, USA; SR 48,968 (gift of Sanofi Récherche, Montpellier, France).

4.2.3. Statistical analyses

Data from ELISA were analyzed with SoftMax®, Microsoft Excel® and Mathematica® softwares. Inhibition curves were fitted using non-linear regression and compared via 95% confidence limits. IC50s, with 95% confidence limits, were calculated from the curves. The Mann-Whitney rank sum test and Kruskal-Wallis ANOVA on ranks were used to test the differences among the inhibition curves. Significance was assumed at the 5% level.

4.3. RESULTS

4.3.1. Relative affinities of α-SP MAb for SP, its fragments and selective NKR antagonists

Using ELISA, a dilution curve was established using a standardized amount of SP-BSA conjugate to capture different dilutions of α-SP MAb (Fig. 4.1). From this, it was determined that a 1:1000 dilution of α-SP MAb solution was suitable for the inhibition ELISA and 25 μL/well of this was used in all assays. The relative affinity of the α-SP MAb for SP, SP fragments and selective NKR antagonists vs the constant amount of SP-BSA conjugate coated onto the plates was estimated by adding known amounts of SP, SP fragments, or selective NKR antagonists to wells and adding the fixed amount of α-SP MAb. Findings from this inhibition ELISA are summarized in Table 4.2 . SP^{1-4}, SP^{1-7} and SP^{1-9}, methionine, the dipeptides, Phe-Phe, Phe-Gly, Gly-Leu and Leu-Met and the selective NKR antagonists (NK1: CP 96,345; NK2: SR 48,968) did not alter α-SP MAb binding (Figs. 4.2-4.4). By contrast, SP and its C-terminal fragments containing $\geq$3 amino acids prevented α-SP MAb binding. The relative affinities of SP and its fragments were obtained from the inhibition curves. Relative affinity of α-SP MAb for SP and SP fragments are summarized in Table 4.2. Interestingly, SP^{6-11} and SP^{7-11} were 250 and 40 times more potent than SP itself at preventing binding, respectively (Fig. 4.5). However, SP^{5-11}, SP^{4-11}, SP^{3-11} and SP^{2-11} were approximately as active as SP (Fig. 4.6); SP^{8-11} and SP^{9-11} were less active than SP at preventing binding (Fig. 4.7).

TABLE 4.2

Relative affinities of α-SP MAb for SP and SP fragments

Amino acid sequence												Abbreviation	Relative affinity
1	2	3	4	5	6	7	8	9	10	11			
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	$CONH_2$	SP	1
								Gly	Leu	Met	$CONH_2$	SP^{9-11}	4×10^{-4}
							Phe	Gly	Leu	Met	$CONH_2$	SP^{8-11}	7.8×10^{-3}
						Phe	Phe	Gly	Leu	Met	$CONH_2$	SP^{7-11}	4.0×10^{1}
					Gln	Phe	Phe	Gly	Leu	Met	$CONH_2$	SP^{6-11}	2.5×10^{2}
				Gln	Gln	Phe	Phe	Gly	Leu	Met	$CONH_2$	SP^{5-11}	6.3×10^{-1}
			Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	$CONH_2$	SP^{4-11}	6.7×10^{-1}
		Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	$CONH_2$	SP^{3-11}	1.2
	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	$CONH_2$	SP^{2-11}	8.3×10^{-1}
Arg	Pro	Lys	Pro	$CONH_2$								SP^{1-4}	$<4.8\times10^{-6}$
Arg	Pro	Lys	Pro	Gln	Gln	Phe	$CONH_2$					SP^{1-7}	$<4.8\times10^{-6}$
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	$CONH_2$			SP^{1-9}	$<4.8\times10^{-6}$

4.3.2. Alanine scanning

Solid phase peptide syntheses on membranes were used to try and confirm the conclusions drawn from measurements of relative affinities by inhibition ELISA. Analogues of SP^{6-11} were prepared in which each amino acid was replaced with an alanine. Table 4.1 shows the amino acid sequences of the peptides used in this study. The activities of these peptides were estimated by dot immunoassay using monoclonal α-SP Ab and polyclonal α-SP serum. Colour was developed in dots with 4-chloro-1-naphthol as TM-Blotting™ failed to yield any color. Using the former reagent, none of the analogues showed any evidence of binding with α-SP MAb, but all showed evidence of binding to polyclonal α-SP serum.

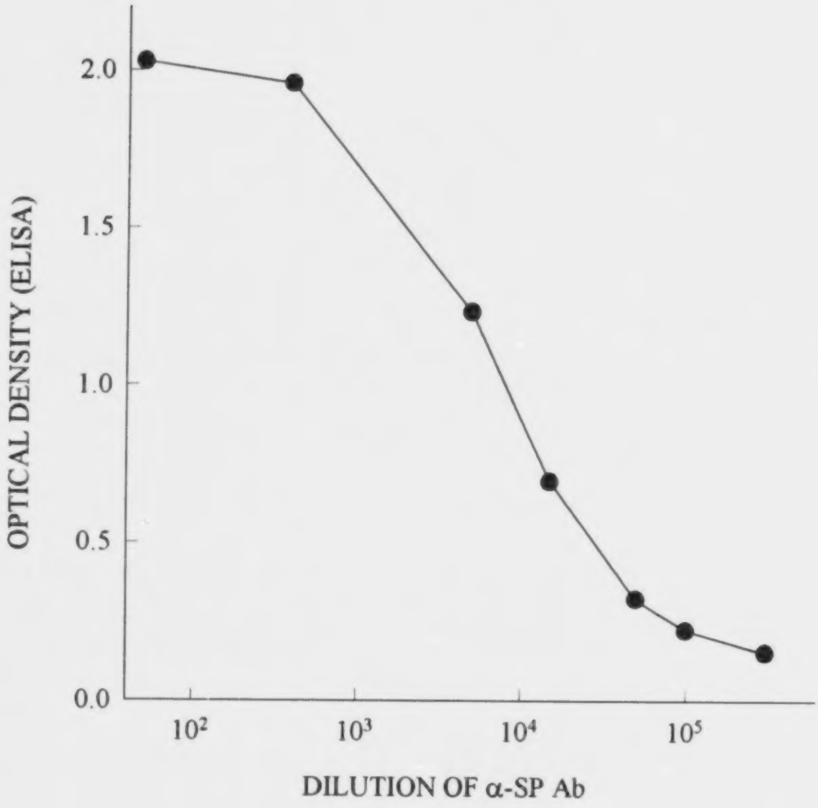


Fig. 4.1

Dilution curve for the anti-substance P antibody obtained using a constant, predetermined amount of SP-BSA conjugate. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

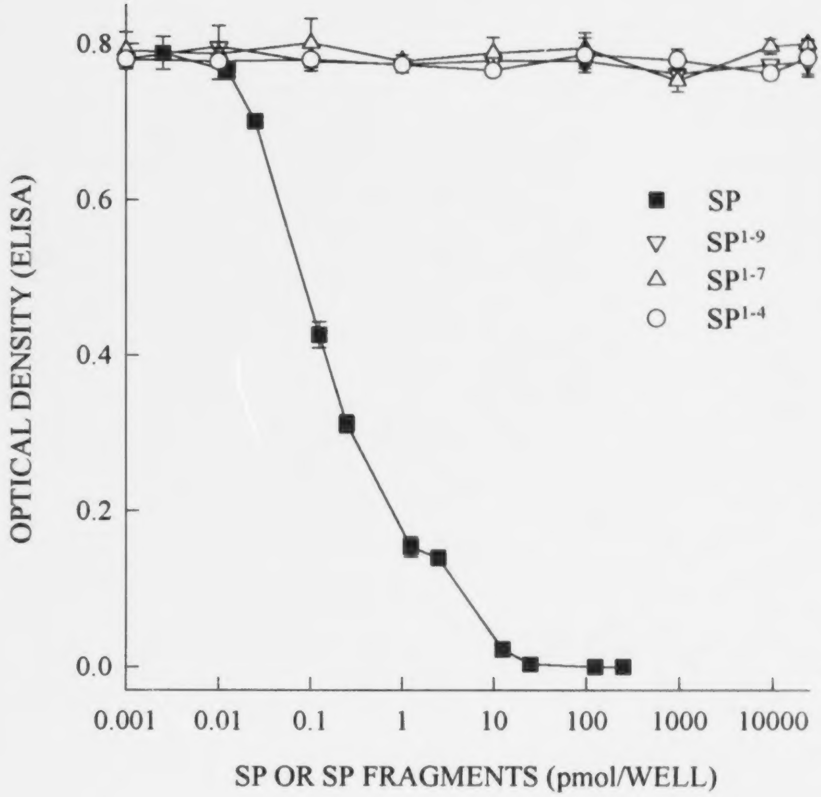


Fig. 4.2

Relative affinities of anti-substance P antibody evaluated with a competitive binding technique in the presence of a predetermined amount of SP-BSA conjugate, 1:1,000 dilutions of α-SP MAb (25 μl) and different concentrations of SP or N-terminal fragments of SP. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

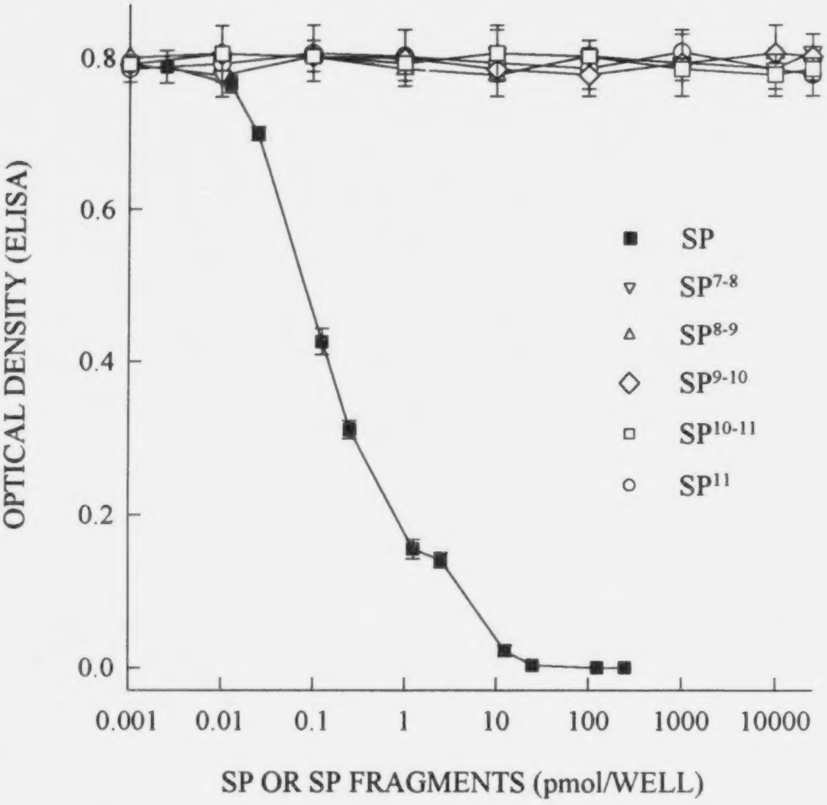


Fig. 4.3

Relative affinities of anti-substance P antibody evaluated with a competitive binding technique in the presence of a predetermined amount of SP-BSA conjugate, 1:1,000 dilutions of α-SP MAb (25 μl) and different concentrations of SP or SP^{7-8}, SP^{8-9}, SP^{9-10}, SP^{10-11} and SP^{11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

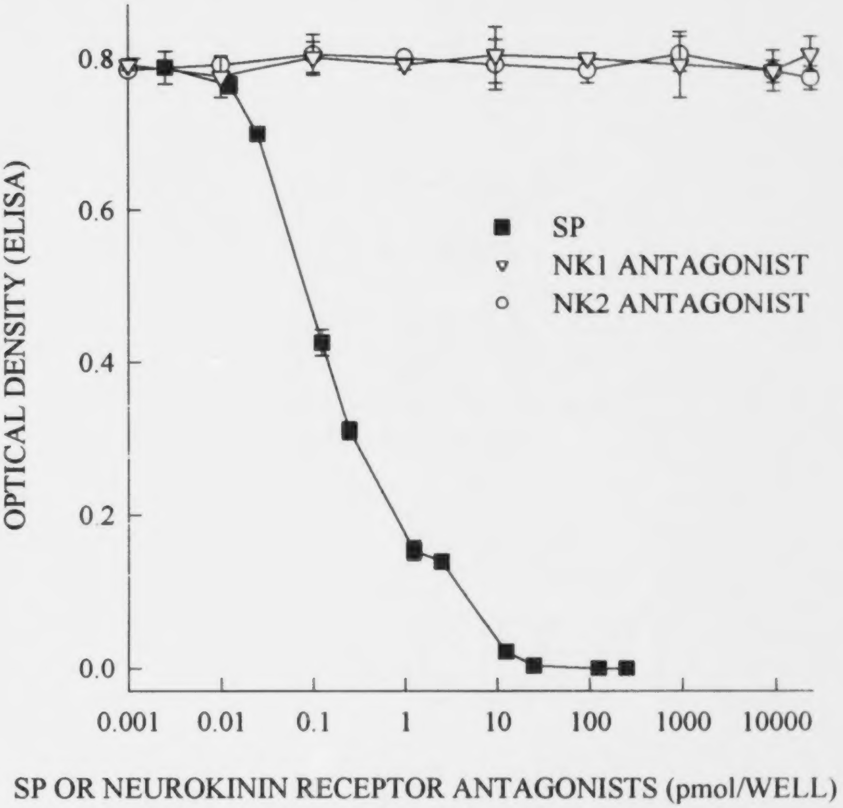


Fig. 4.4

Relative affinities of anti-substance P antibody evaluated with a competitive binding technique in the presence of a predetermined amount of SP-BSA conjugate, 1:1,000 dilutions of α-SP MAb (25 μl) and different concentrations of SP or selective tachykinin receptor antagonists. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

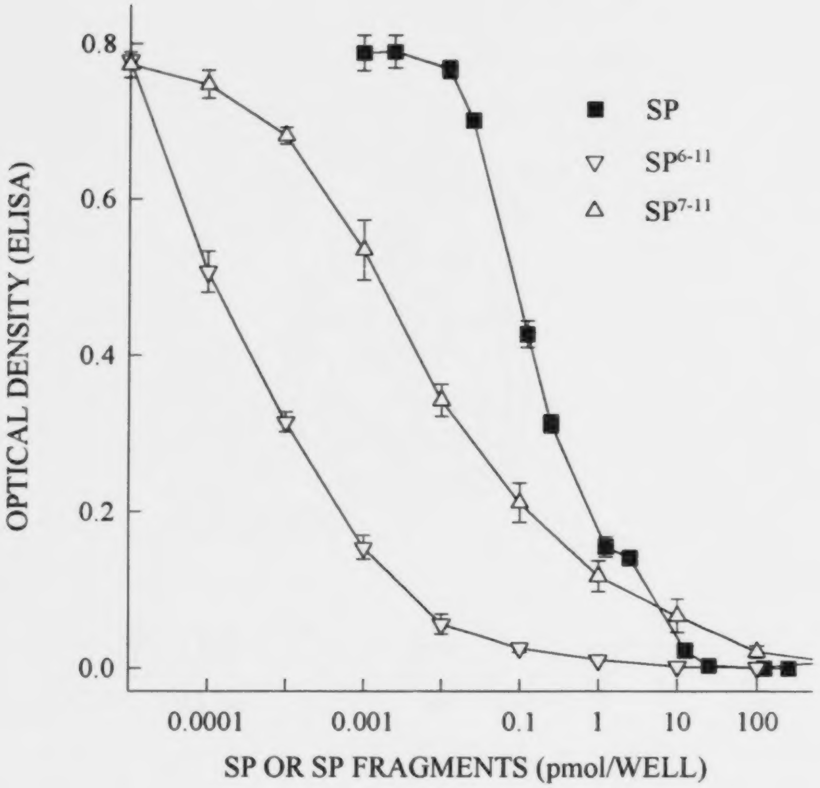


Fig. 4.5

Relative affinities of anti-substance P antibody evaluated with a competitive binding technique in the presence of a predetermined amount of SP-BSA conjugate, 1:1,000 dilutions of α-SP MAb (25 μl) and different concentrations of SP or SP$^{6-11}$ and SP$^{7-11}$. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

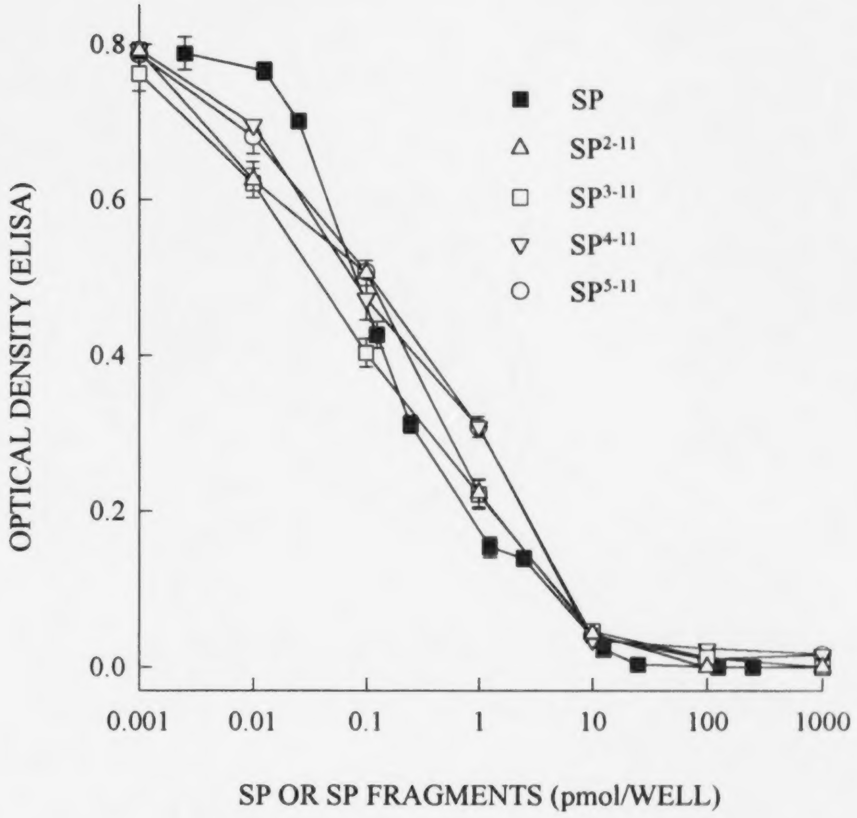


Fig. 4.6

Relative affinities of anti-substance P antibody evaluated with a competitive binding technique in the presence of a predetermined amount of SP-BSA conjugate, 1:1,000 dilutions of α-SP MAb (25 μl) and different concentrations of SP or SP^{2-11}, SP^{3-11}, SP^{4-11} and SP^{5-11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

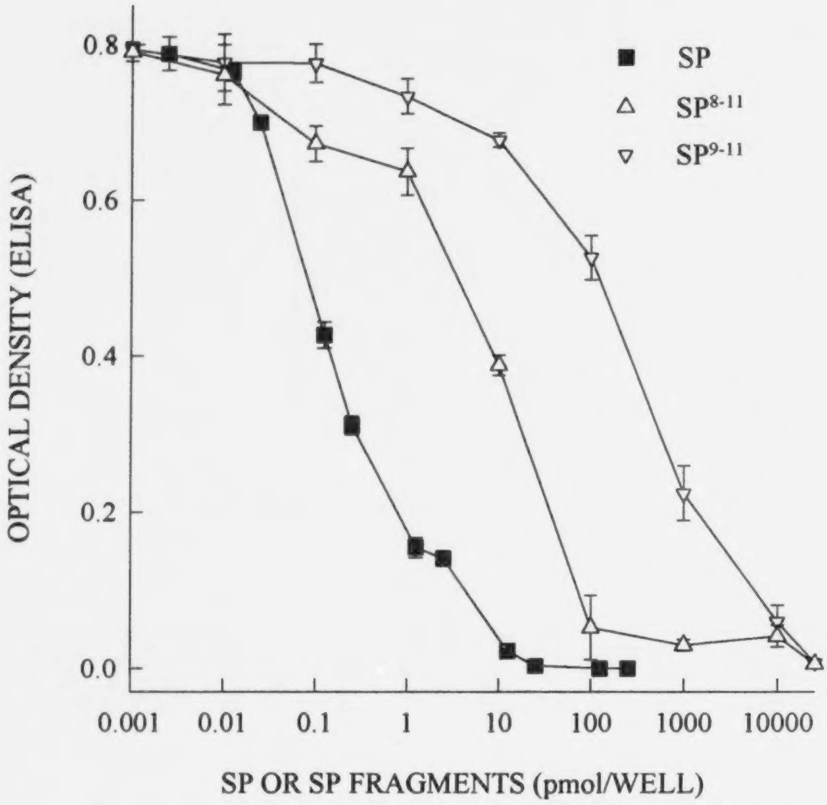


Fig. 4.7

Relative affinities of anti-substance P antibody evaluated with a competitive binding technique in the presence of a predetermined amount of SP-BSA conjugate, 1:1,000 dilutions of α-SP MAb (25 μl) and different concentrations of SP or SP^{8-11} and SP^{9-11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

4.4. DISCUSSION

The relative affinities of the α-SP MAb for SP and its various fragments were estimated by inhibition ELISA. In this competitive binding technique, a constant amount of SP-BSA conjugate bound to the plate was used to capture α-SP MAb that had not bound to different concentrations of SP or its fragments in solution (Jafarian et al., 1995). The amount of α-SP MAb added was determined from a dilution curve and kept constant in all experiments. This ensured reproducible measurements of relative affinity. The findings indicated that the α-SP MAb had approximately the same affinity for SP^{2-11}, SP^{3-11}, SP^{4-11} and SP^{5-11} as SP itself. By contrast, the α-SP MAb's affinity for SP^{6-11} and SP^{7-11} was respectively 250 and 40 times greater than for SP. However, α-SP MAb's affinity for SP^{8-11} and SP^{9-11} was less than that for SP. In agreement with earlier findings (Cuello et al, 1979), the data indicated that α-SP MAb is directed against C-terminus of SP. Moreover, amino acids 6 and 7, and, to a lesser extent, amino acids 8 and 9, play a crucial role in determining the α-SP MAb's affinity for SP and its fragments. Interestingly, in this system, the α-SP MAb showed no affinity for N-terminal SP fragments and mono- or di-peptides derived from SP's C-terminal, confirming the crucial role of all the four amino acids in the C-terminal of SP in determining affinity. The NC1/34 was generated by immunizing mice with an antigen prepared by using glutaraldehyde to couple SP via its N-terminal to keyhole limpet hemocyanin. Thus, the α-SP MAb should be directed towards an epitopic site on the C-terminal part of the molecule. Similarity in the C-terminus of TK could account for weak cross reactivity of α-SP MAb with eleidosin and NKA reported previously (Cuello et al., 1979; Jafarian et al., 1995). It is possible that conjugating SP to BSA and absorbing it onto the plate alters or impedes expression of its relevant epitopic sites. Thus, the conjugate's ability to compete for α-SP MAb with free SP or SP fragments is probably reduced. Thus, this is not a true "competitive" inhibition assay. However, as the conditions were similar for SP and SP fragments, it is likely that the values obtained for the α-SP MAb's relative affinities for them are representative. It was noteworthy that the α-SP MAb's affinity decreased from a peak at SP^{6-11} to values around that for SP for fragments SP^{5-11}, SP^{4-11}, SP^{3-11} and SP^{2-11}. This difference may be related to the fragments' molecular size or changes in their conformation in solution. SP^{6-11} and NKA^{6-11} differ by two amino acids at positions 8 and 6 - Phe *vs* Val; Gln *vs* Ser, respectively. The commonality at amino acids 7 (Phe *vs* Phe) and 9 (Gly *vs* Gly) may contribute to the cross reactivity observed.

The use of monoclonal antibodies against TK appears to be a powerful approach for detecting TK and investigating their role. However, similarity in the C-terminus of TK may preclude the use of monoclonal antibodies directed towards the C-terminus of TK. Thus, to detect and differentiate between the effects of TK molecules, it is necessary to use highly specific monoclonal antibodies with minimal cross reactivity with other related peptides. Antibodies directed against the respective N-terminals, where there is considerable variability among TK should be more specific.

De novo peptide synthesis was used to try and confirm the conclusions drawn from measurements of relative affinities by inhibition ELISA. There are two different ways to create a defined set of peptide sequences: the genetic and the synthetic approach (Fields & Noble, 1990). The latter approach was adopted for these studies. It was decided to use the SPOTs solid phase synthesis system. With this method, numerous peptides can be synthesized simultaneously on a derivatized cellulose membrane. Peptide "SPOTs" can then be evaluated using a dot immunoassay. Analogues of SP^{6-11} were prepared with single sequential substitutions of alanine for each amino acid (Table 4.1). As SPOTs syntheses utilized the N-terminal for the addition of amino acids to the peptide, similar peptides in which the C-terminal was "spaced" away from the membrane by four arginine molecules to expose the methionine residue were also prepared. Lastly, analogues of SP were prepared in which the C- and N-terminals were reversed. Dot immunoassay of all these peptides revealed that none of these peptides bound the monoclonal α-SP Ab, but all of them bound the polyclonal α-SP serum used as a positive control. These findings indicate the importance of an accessible and untreated C-terminal portion of the peptide for binding to the α-SP MAb. Addition of spacer arginine molecules clearly did not provide this. The SPOTs system used the C-terminal to bind the peptide to the membrane and added amino acids via the N-terminal. Since the C-terminal is an important contributor to the α-SP MAb's affinity for peptides, this method and alanine replacement studies unfortunately did not provide information about the role of specific amino acids in binding to α-SP MAb.

Lastly, we attempted to determine whether there were any similarities between the α-SP MAb's paratopic site(s) and NKR by measuring the Ab's affinity for the selective NK1-receptor antagonist CP 96,345 (Sinder et al., 1991) and the selective NK2-receptor antagonist SR 48,968 (Emonds-Alt et al., 1992). NK1 and NK2 receptors belong to the family of G-protein-coupled receptors. Neurokinin agonists and antagonists bind to the receptors' extracellular domains (Frossard & Advenier, 1991; Cascieri et al., 1992). The structure-activity relationships involved in SP's binding have been much studied (Regoli et al., 1984a; Buck & Burcher, 1986; Laufer et al., 1986). The smallest fragment with significant affinity for NKR is SP^{6-11} (Regoli et al., 1984b; Wang et al., 1993a; Boyle et al., 1994). Interestingly, Wang et al. (1993b) showed that changes of chirality of the amino acids in the C-terminal of SP markedly altered affinity for the NK1-receptor. By contrast, changes at the N-terminal had much less effect. In our experiments, the α-SP MAb bound neither the NK1- nor the NK2-receptor antagonist suggesting that the paratopic site on the antibody bears little resemblance to the binding sites on NKR.

In conclusion, these experiments suggest that the monoclonal α-SP MAb derived from the rat/mouse heterohybridoma NC1/34 is directed against epitopic sites on SP's C-terminal. Amino acids 6 and 7, and, to a lesser extent, 8 and 9, are important in determining affinity. It appears that the methionine residue in the C-terminal of SP and its fragments has to be exposed for binding to occur. However, we were unable to confirm these conclusions using alanine scanning probably because the peptides synthesized were attached to the SPOTs membrane via their C-terminal. In retrospect, it would have been better to have employed a method of synthesis for the peptides that used the N-terminal for addition of each amino acid.

CHAPTER 5

PHARMACOLOGIC CHARACTERIZATION OF BRONCHOSPASM INDUCED BY SUBSTANCE P AND SUBSTANCE P FRAGMENTS IN GUINEA-PIGS

A version of this chapter has been submitted to Pulmonary Pharmacology for publication.

Jafarian A. and Biggs. D.F.

5.1. INTRODUCTION

SP is a neuropeptide considered to function as a neurotransmitter or neuromodulator in the central and peripheral nervous system (James et al., 1992). It is well characterized in terms of sites and mechanisms of biosynthesis, distribution, site of release and biological actions. SP belongs to a family of closely-related peptides known as TK, that are widely distributed in the airways and lungs of several species, including humans and guinea-pigs (Maggio, 1985; Guard & Watson, 1991; Maggi, 1993; Maggi et al., 1993). Given parenterally, they induce a variety of responses including contraction of bronchial smooth muscle, mucus secretion, vasodilation, extravasation of plasma proteins and recruitment of inflammatory cells (Lundberg & Saria, 1987; Maggi, 1990). These effects comprise "neurogenic" inflammation. It has been suggested that release of endogenous TK, SP and NKA, from pulmonary afferent C-fibres contributes to bronchial obstruction in asthma (Lundberg & Saria, 1987; Maggi, 1990; Maggi, 1993).

With few, but important exceptions (e.g. mast cell degranulation and some behavioral effects), the biological effects of TK are mediated via their structurally similar carboxy-terminal sequence: ....Phe-X-Gly-Leu-Met-$CONH_2$ on NK1, NK2 and NK3 receptors (Erspamer, 1981; Guard & Watson, 1991; Nakanishi, 1991; Gerard et al., 1993). Predictably, *in vitro* and *in vivo*, non-selective or selective NKR antagonists reduced or abolished tachykinin-induced bronchospasm (Ballati et al., 1992; Ichinose et al., 1992; Foulon et al., 1993; Maggi, 1993).

As with other peptide transmitters, the physiologic effects of TK are limited by enzymatic degradation at or near their site of release. All TK are subject to cleavage by

NEP; SP is also subject to cleavage by ACE and aminopeptidase (Lilly et al., 1993). Studies *in vitro* have shown that ACE hydrolyses SP at Gly^{9}-Leu^{10} and Phe^{8}-Gly^{9}. NEP cleaves SP preferentially at the amino-terminal side of the hydrophobic amino acid residues Phe^{7} or Phe^{8} and Leu^{10}. Some of the metabolites are further cleaved by these enzymes (Lilly et al., 1993). *In vivo*, the major metabolites of SP are SP$^{1-9}$ and SP$^{10-11}$ (Masata et al., 1984; Borson et al., 1987). *In vitro* and *in vivo*, NEP or ACE inhibitors have been shown to augment SP and NKA's biologic effects (Borson et al., 1987; Shore & Drazen, 1988; Shore & Drazen, 1991). The products of NEP and ACE cleavage of SP at Gly^{9}-Leu^{10} have no bronchospastic activity in guinea-pigs, but SP$^{3-11}$ and SP$^{5-11}$, which result from cleavage of SP by dipeptidyl (amino) peptidase IV, are more potent bronchoconstrictors than SP (Shore & Drazen, 1988). We used selective NK1 and NK2 receptor antagonists to characterize the bronchospasm induced in anesthetized guinea-pigs by injection of SP or its fragments.

5.2. METHODS AND MATERIALS

5.2.1. Animals

SPF-quality, female Hartley-strain guinea-pigs (weight range: 350-450 g) were obtained from Charles River Inc., St. Constant, Québec. They were transported to Edmonton in filter-top boxes and housed in laminar flow units (Bioclean™, Hazleton) on grids over trays of rock salt and fed guinea-pig chow supplemented with apples. Water was allowed *ad lib.*

5.2.2. Methods

5.2.2.1. Measurement of airways' responsiveness

Groups (n = 3-5) of SPF-quality, female, Hartley-strain guinea-pigs (weight range: 400-450 g) were used. They were anesthetized with sodium pentobarbital (40-50 mg kg^{-1}, ip, with additional doses, 5 mg kg^{-1}, iv, as required). Their tracheas were cannulated (PE240) and artificial respiration was applied (tidal volume = 9 ml kg^{-1}, pump speed = 20 strokes min^{-1}) with a rodent ventilator (Ugo Basile, Varese, Italy). A jugular vein was cannulated (PE50) for giving drugs iv. Succinylcholine (0.03 mg kg^{-1}, iv) was given to paralyse animals and prevent spontaneous respiratory movements. Pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L) were measured continuously, breath-by-breath, using a computerized system (Jafarian et al., 1995). Agonists were given in ascending order of dose and after each dose animals were inflated with two tidal volumes to expedite return to baseline values of R_L and E_L. Dose-response (peak response as percent change from baseline values of R_L and E_L) curves to SP, SP^{9-11}, SP^{8-11}, SP^{7-11}, SP^{6-11}, SP^{5-11}, SP^{4-11}, SP^{3-11}, SP^{2-11}, SP^{1-4}, SP^{1-7} and SP^{1-9} were established and the effects of selective NK1 or NK2 receptor antagonist were determined. The amino acid sequences of the peptides used are shown in Table 4.2. The relative bronchoconstrictor activities of SP and its fragments were estimated by linear regression. Antagonists were given iv 15 min before injecting SP or SP fragments. Drugs were dissolved in distilled water except for SR 48,968 (4 mg was dissolved in ethanol 95%, 0.1 ml), SP^{6-11} (dimethylsulfoxide, 0.1 ml) and SP^{1-7} and SP^{4-11} (acetic acid, 0.05%). All drugs were diluted to their final concentrations with 0.9% saline.

5.2.3. Materials

Drugs used were: substance P (Biochem Pharma Inc., Montréal, PQ), succinylcholine chloride, SP^{9-11}, SP^{8-11}, SP^{7-11}, SP^{6-11}, SP^{5-11}, SP^{4-11}, SP^{3-11}, SP^{2-11}, SP^{1-4}, SP^{1-7} and SP^{1-9} (Sigma, St. Louis, MO USA), sodium pentobarbital (Euthanyl™, M.T.C Pharmaceuticals, Markham, ON); methionine, Phe-Phe, Phe-Gly, Gly-Leu and Leu-Met (Bachem Bioscience Inc., Philadelphia, PA USA); CP 96,345 and CP 99,994 were gifts of Pfizer Inc., Groton, CO. SR 48,968 was gift of Sanofi Récherche, Montpelier, France.

5.2.4. Statistical analyses

Dose-response curves were plotted as mean ± SEM. Data were analyzed using Sigmastat™ and SAS programs. Mann-Whitney rank sum tests, Student's *t* tests and one-way ANOVA were used to examine differences among responses; least square linear regression analyses were used to determine linearity and relative potency of peptides. Differences among ED_{700} and ED_{250} (nmol kg^{-1} of peptide required to cause 700% increase in R_L, or 250% increase in E_L, respectively) were used to compare peptides' potency. Significance was assumed at the 5% level.

5.3. RESULTS

5.3.1. Bronchopulmonary responses to SP and SP fragments

N-terminal fragments of SP and C-terminal fragment of SP containing < 6 amino acids had no bronchospastic effects in doses up to 3.5 μmol kg^{-1}. SP (1.49 - 5.94 nmol kg^{-1}), SP^{2-11} (1.68-6.71 nmol kg^{-1}), SP^{3-11} (1.83-7.31 nmol kg^{-1}), SP^{4-11} (0.041-0.828 nmol

kg^{-1}), SP^{5-11} (1.15-11.50 nmol kg^{-1}) and SP^{6-11} (1.35-10.80 nmol kg^{-1}), all iv, induced dose-dependent increases in R_L and E_L (for R_L: $r^2 = 0.9594-0.9983$; for E_L: $r^2 = 0.8997-0.9742$) (Figs. 5.1-5.2). On plotting maximal (peak) responses to individual doses, the bronchoconstrictor activities of SP and SP fragments were compared by linear regression. ED_{700} (R_L) and ED_{250} (E_L) were determined from the dose-response curves and used to compare the relative bronchoconstrictor activity of these peptides. Data are summerized in Table 5.1 The order of potency of SP and SP fragments was: $SP^{4-11} >> SP^{5-11} = SP^{3-11} = SP^{2-11} > SP^{6-11} = SP >>> SP^{7-11} > SP^{8-11}$ (Figs. 5.1-5.2 and Table 5.1).

5.3.2. Effects of NKR antagonists on bronchopulmonary responses induced by SP and SP fragments

Pharmacologic characterization of the bronchoconstrictor activity of SP and SP fragments was carried out with selective NK1 and NK2 receptor antagonists. The selective NK1 receptor antagonists, CP 96,345 or CP 99,994 (3.0 mg kg^{-1}, iv), and the selective NK2 receptor antagonist, SR 48,968 (1.0 mg kg^{-1}, iv), did not alter baseline values of R_L and E_L ((R_L: before = 0.27 ± 0.03; after = 0.27 ± 0.03; n = 38, $p > 0.05$); (E_L: before = 1.75 ± 0.29; after = 1.74 ± 0.31; n = 38, $p > 0.05$)). We have shown that these doses of CP 96,345 and SR 48,968 were effective in decreasing the bronchopulmonary responses to SP, NKA and selective NK1 or NK2 receptor agonists (se chapter 6 for details). The selective NK1 receptor antagonists caused a significant rightward shift of the dose-response curves to SP and SP fragments (n = 3-5, $p < 0.05$) (Figs. 5.3-5.8, Table 5.1). By contrast, the selective NK2 receptor antagonist had no significant effect on bronchospastic

TABLE 5.1

The relative bronchoconstrictor activities of substance P (SP) and SP fragments

Agonist	n	ED_{700} (R_L) (nmol kg^{-1})	Relative potency	ED_{250} (E_L) (nmol kg^{-1})	Relative potency
SP	5	5.81 ± 0.94	1.00	4.23 ± 0.51	1.00
SP^{2-11}	3	3.94 ± 0.63	1.48	2.42 ± 0.43	1.75
SP^{3-11}	3	3.80 ± 0.67	1.53	2.38 ± 0.52	1.77
SP^{4-11}	3	0.15 ± 0.03	38.73	0.47 ± 0.09	9.00
SP^{5-11}	3	3.85 ± 0.44	1.63	2.66 ± 0.61	1.59
SP^{6-11}	3	3.58 ± 0.74	1.03	4.05 ± 0.76	1.04
SP^{7-11}	3	> 2500	< 0.002	> 2500	< 0.002
SP^{8-11}	3	> 2500	< 0.002	> 2500	< 0.002
SP^{9-11}	3	> 5000	< 0.001	> 5000	< 0.001
SP^{10-11}	3	> 5000	< 0.001	> 5000	< 0.001
SP^{11}	3	> 5000	< 0.001	> 5000	< 0.001
SP^{7-8}	3	> 5000	< 0.001	> 5000	< 0.001
SP^{8-9}	3	> 5000	< 0.001	> 5000	< 0.001
SP^{9-10}	3	> 5000	< 0.001	> 5000	< 0.001

Values are means ± STD; n, number of experiments; ED_{700} (R_L), doses of peptides (nmol kg^{-1}) required to cause 700% increase in pulmonary flow resistance (R_L) from baseline values. ED_{250} (E_L), doses of peptides (nmol kg^{-1}) required to cause 250% increase in dynamic pulmonary elastance (E_L) from baseline values.

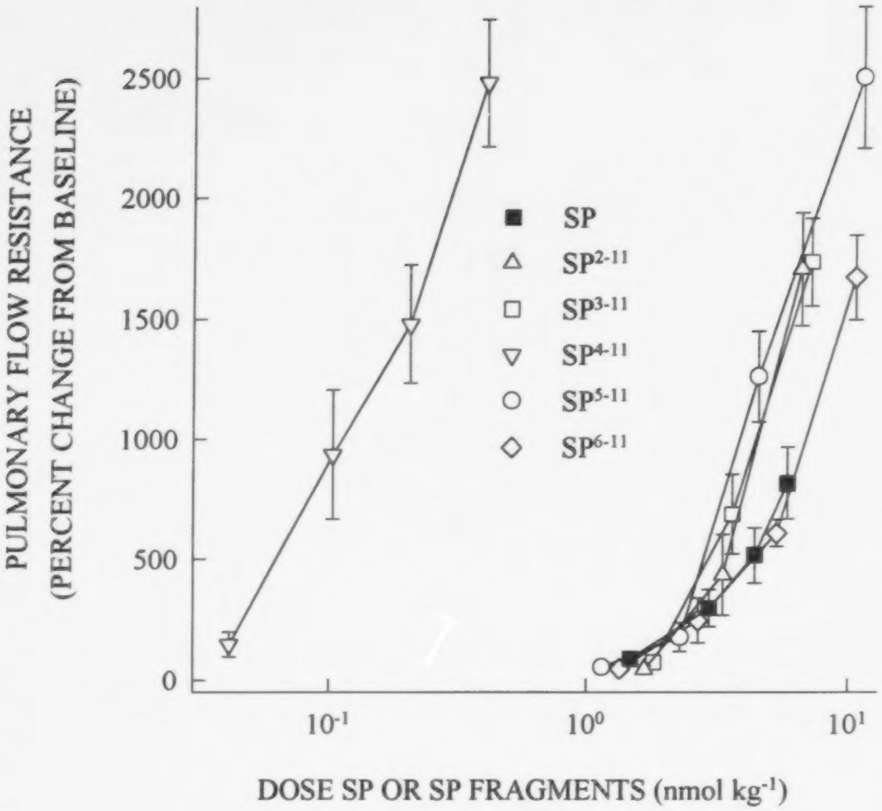


Fig. 5.1

Changes in pulmonary flow resistance in response to various doses of SP and SP fragments (all iv) in anesthetized, paralyzed guinea-pigs. Results, expressed as percent changes from baseline values, are mean ± SEM of 5 experiments.

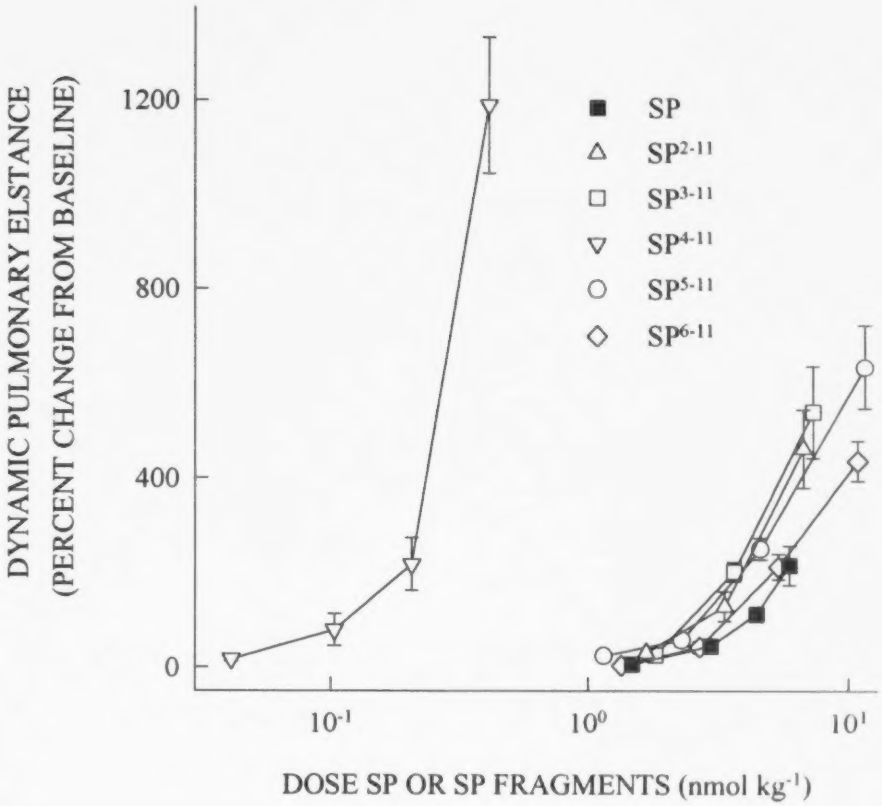


Fig. 5.2

Changes in dynamic pulmonary elastance in response to various doses of SP and SP fragments (all iv) in anesthetized, paralyzed guinea-pigs. Results, expressed as percent change from baseline values, are mean ± SEM of 5 experiments.

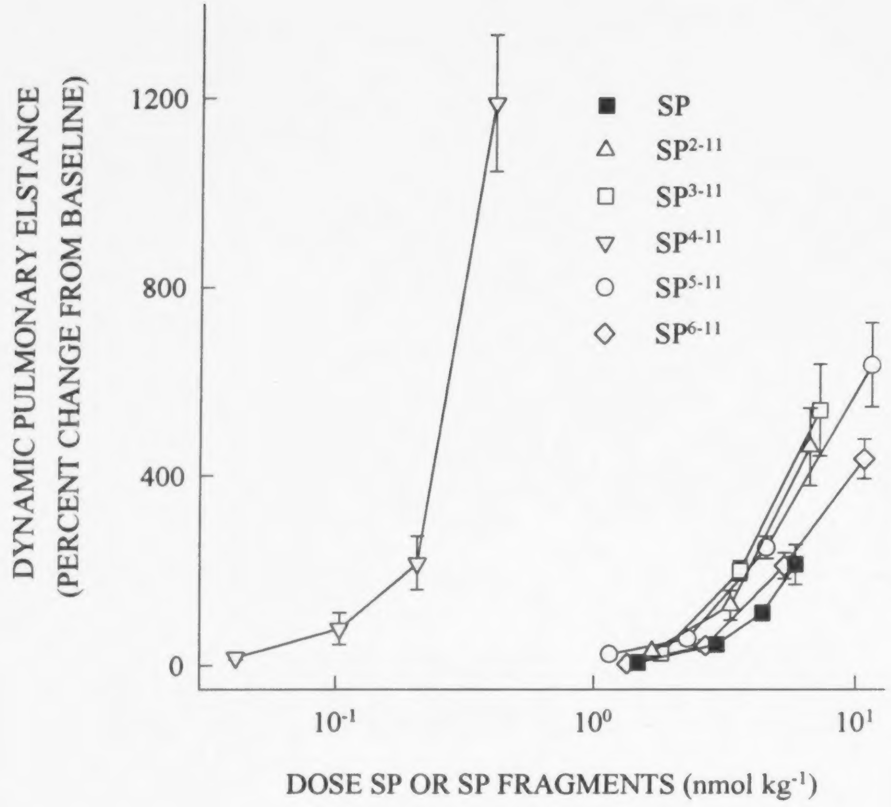


Fig. 5.3

The effects of iv administration of 3 mg kg^{-1} CP 96,435 (selective NK1 receptor antagonist), 1 mg kg^{-1} SR 48,968 (selective NK2 receptor antagonist) or combination of 3 mg kg^{-1} CP 96,435 and 1 mg kg^{-1} SR 48,968 on the changes in pulmonary flow resistance induced by various doses of SP iv. Results, expressed as percent changes from baseline values, are mean ± SEM of 5 experiments.

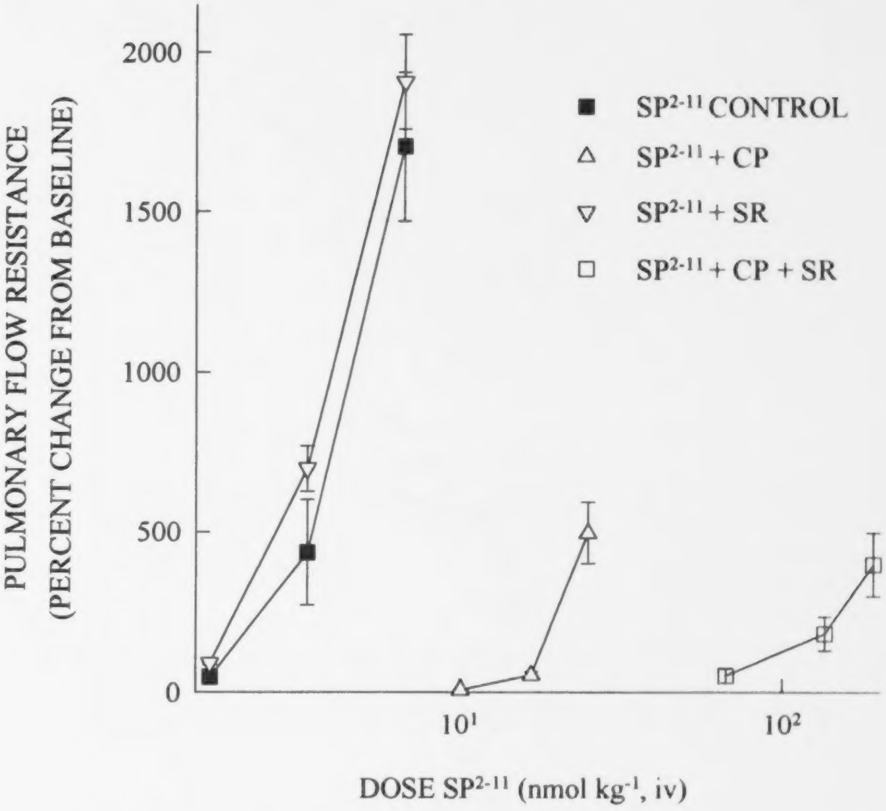


Fig. 5.4

The effects of iv administration of 3 mg kg^{-1} CP 99,994 (selective NK1 receptor antagonist), 1 mg kg^{-1} SR 48,968 (selective NK2 receptor antagonist) or combination of 3 mg kg^{-1} CP 99,994 and 1 mg kg^{-1} SR 48,968 on the changes in pulmonary flow resistance induced by various doses of SP$^{2-11}$ iv. Results, expressed as percent changes from baseline values, are mean ± SEM of 3 experiments.

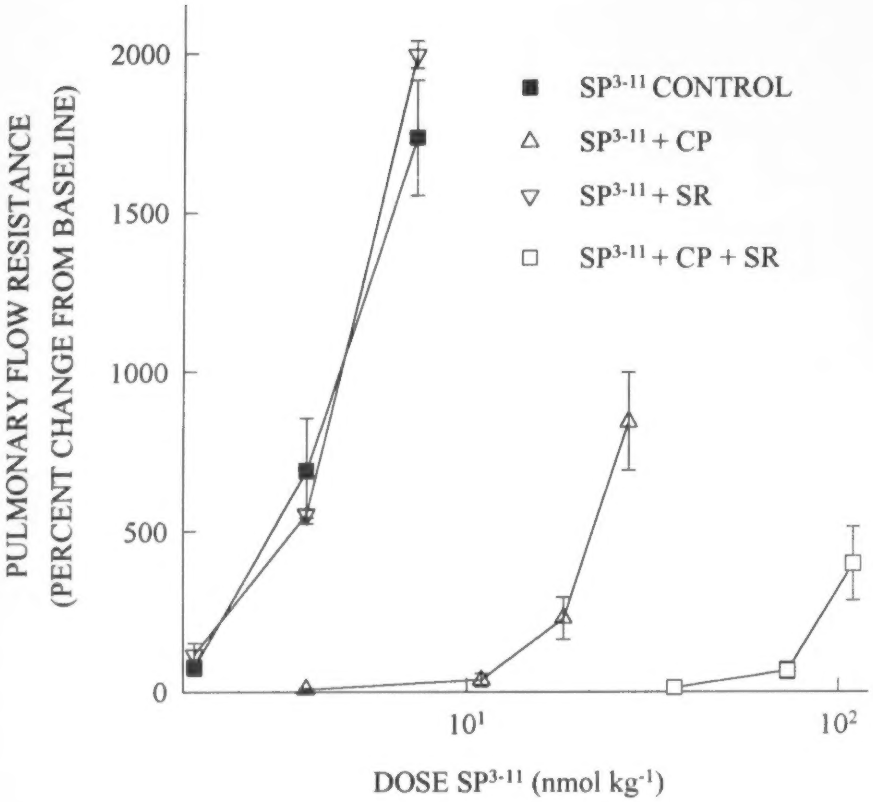


Fig. 5.5

The effects of iv administration of 3 mg kg^{-1} CP 99,994 (selective NK1 receptor antagonist), 1 mg kg^{-1} SR 48,968 (selective NK2 receptor antagonist) or combination of 3 mg kg^{-1} CP 99,994 and 1 mg kg^{-1} SR 48,968 on the changes in pulmonary flow resistance induced by various doses of SP$^{3\text{-}11}$ iv. Results, expressed as percent changes from baseline values, are mean ± SEM of 3 experiments.

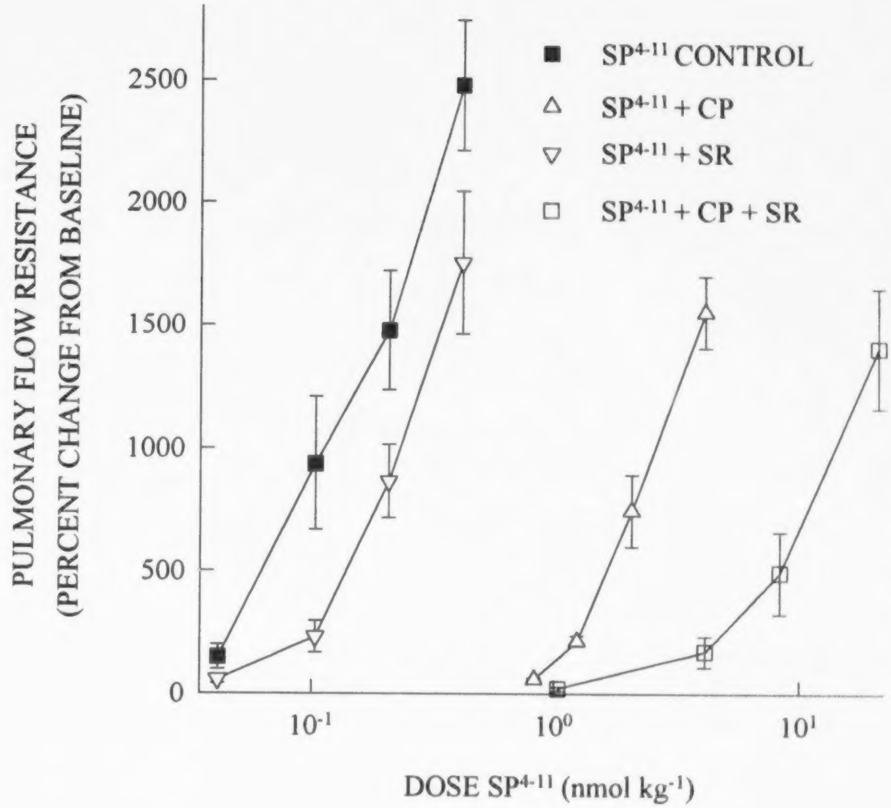


Fig. 5.6

The effects of iv administration of 3 mg kg^{-1} CP 96,435 (selective NK1 receptor antagonist), 1 mg kg^{-1} SR 48,968 (selective NK2 receptor antagonist) or combination of 3 mg kg^{-1} CP 96,435 and 1 mg kg^{-1} SR 48,968 on the changes in pulmonary flow resistance induced by various doses of SP$^{4-11}$ iv. Results, expressed as percent changes from baseline values, are mean ± SEM of 3 experiments.

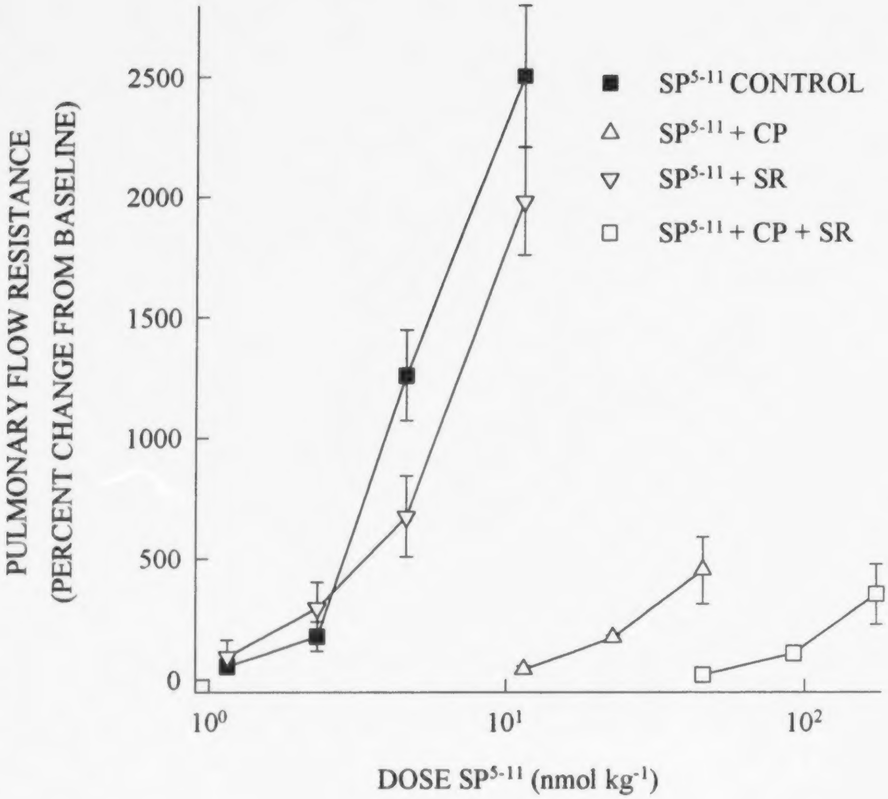


Fig. 5.7

The effects of iv administration of 3 mg kg^{-1} CP 96,435 (selective NK1 receptor antagonist), 1 mg kg^{-1} SR 48,968 (selective NK2 receptor antagonist) or combination of 3 mg kg^{-1} CP 96,435 and 1 mg kg^{-1} SR 48,968 on the changes in pulmonary flow resistance induced by various doses of SP$^{5-11}$ iv. Results, expressed as percent changes from baseline values, are mean ± SEM of 3 experiments.

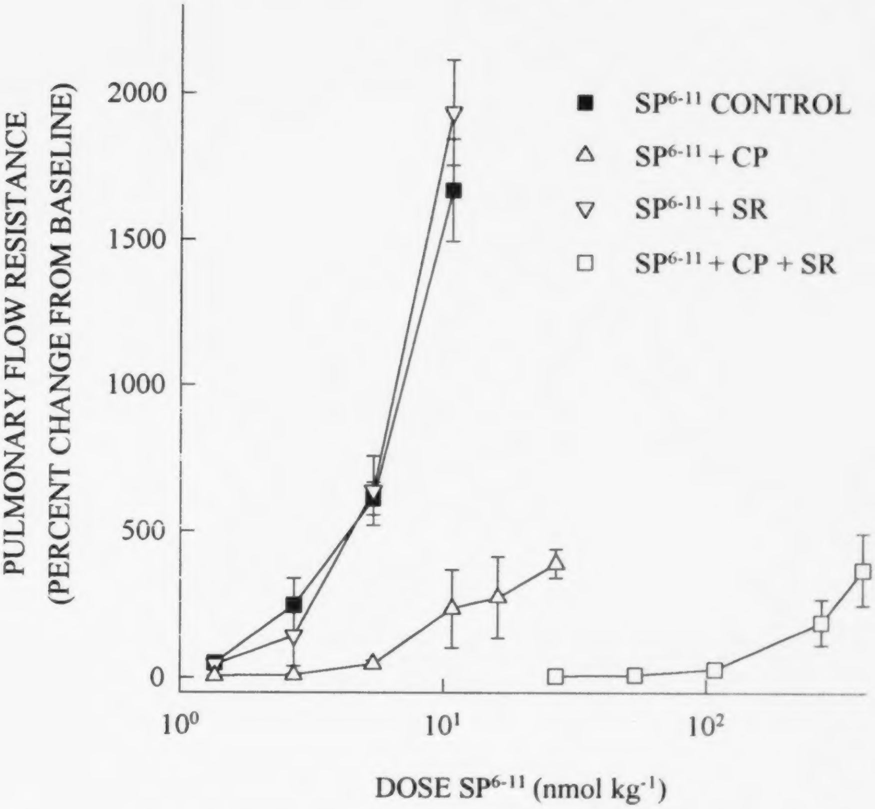


Fig. 5.8

The effects of iv administration of 3 mg kg^{-1} CP 96,435 (selective NK1 receptor antagonist), 1 mg kg^{-1} SR 48,968 (selective NK2 receptor antagonist) or combination of 3 mg kg^{-1} CP 96,435 and 1 mg kg^{-1} SR 48,968 on the changes in pulmonary flow resistance induced by various doses of SP^{6-11} iv. Results, expressed as percent changes from baseline values, are mean ± SEM of 3 experiments.

responses to SP and all of its C-terminal fragments except for SP^{4-11} ($n = 3$-5, $p > 0.05$) (Figs. 5.3-5.8). However, a combination of the NK1 and NK2 receptor antagonists induced significantly greater rightward shift of dose-response curves to SP and its fragments (Figs. 5.3-5.8). Results are quantitated and summarized in Table 5.2 (ED_{300} (R_L) (dose of agonist required to cause 300% increase in R_L was used to compare the effect of NKR antagonists on SP- and SP fragments-induced bronchospasm because in the presence of these antagonists large quantities of peptides were required to get ED_{700})). The ratios of the doses of agonists required for inducing equivalant changes in R_L in the absence and the presence of selective NKR antagonists were computed (Table 5.2). Pretreatment with NK1 receptor antagonist resulted in a rightward shift (3-9 fold) in the dose-response curves to SP or its fragments. Pretreatment with combination of NK1 and NK2 receptor antagonists resulted in a rightward shift (38-59 fold) in the dose-response curve to SP or its fragments (Table 5.2). Data for E_L are not shown; the findings were similar to those for R_L.

5.4. DISCUSSION

Structure-activity relationship studies showed that amino acid sequence of the C-terminal of SP is necessary for most of its biologic activities (Frossard & Advenier, 1991). In this study we characterized neurokinin-mediated responses in guinea-pigs by comparing the actions of selective NK1 and NK2 receptor antagonists against SP- or SP fragment-induced bronchoconstriction. Our findings

TABLE 5.2

Antagonism of the bronchospastic effects of substance P (SP) and SP fragments by selective NKR antagonists in anesthetized guinea-pigs

Neurokinin antagonists		Control	CP 96,345 or CP 99,994	Ratio	SR 48,968	Ratio	CP 96,345 or CP 99,994 + SR 48,968	Ratio
Treatment	n	ED_{300} (nmol kg^{-1})	ED_{300} (R_L) (nmol kg^{-1})		ED_{300} (R_L) (nmol kg^{-1})		ED_{300} (R_L) (nmol kg^{-1})	
SP	5	2.99 ± 0.91	> 20	-	3.74 ± 0.94	1.25	> 40	-
SP^{2-11}	3	3.95 ± 0.91	17.35 ± 2.21	4.4	4.12 ± 0.89	1.04	173.25 ± 14.93	43.9
SP^{3-11}	3	2.53 ± 0.48	17.72 ± 2.00	7.0	2.58 ± 0.11	1.02	109.12 ± 15.76	43.1
SP^{4-11}	3	0.15 ± 0.03	1.39 ± 0.31	9.2	0.12 ± 0.03	0.80	5.63 ± 0.85	37.5
SP^{5-11}	3	3.86 ± 0.44	34.26 ± 5.31	8.9	4.02 ± 0.53	1.04	221.32 ± 19.43	57.3
SP^{6-11}	3	5.83 ± 0.74	16.83 ± 3.22	2.8	5.99 ± 0.82	1.03	345.73 ± 62.71	59.3

Values are means ± STD; n, number of experiments; ED_{300}, doses of peptides (nmol kg^{-1}) required to cause 300% increase in pulmonary flow resistance (R_L) from baseline values.

indicated that bronchospastic activity of SP depends on the its C-terminal. These findings confirm and extend those of Shore and Drazen (1988) who also showed that iv administration of SP or its C-terminal fragments induced bronchoconstriction in guinea-pigs. The inability of SP's N-terminal fragments to induce bronchospasm further confirms the involvement of the C-terminal of SP in inducing bronchospasm. It has been shown that the methionine at position 11 has a crucial role in determining SP's activity (Chipkin et al., 1979) which may explain the inability of N-terminal fragments of SP to induce bronchospasm.

SP^{6-11} was as potent a bronchoconstrictor as SP but C-terminal fragment of SP containing < 6 amino acids had no specific bronchospastic effect at the doses examined. These findings suggest the minimum requirement for inducing a specific bronchospastic activity is 6 amino acids in the C-terminal of SP. These findings are in agreement with those of Pernow (1983) who showed that SP^{6-11} was the smallest fragment of SP with the ability to induce biologic activities. Removal of up to 4 amino acids from N-terminal of SP increased the bronchoconstrictor activities of these peptides as SP^{2-11}, SP^{3-11}, SP^{4-11} and SP^{5-11} were more potent bronchoconstrictors than SP. However, removal of more than five amino acids reduced or abolished bronchoconstrictor activity. The bronchospastic effect of C-terminal fragments, SP^{7-11} and SP^{8-11}, seen only at high doses, could possibly be due to the low affinity of these peptide for NKR. Also, interaction with non-specific sites may possibly explain the effects of SP^{7-11} and SP^{8-11} since other basic amino acid residues like tuftsin, mastoparan and cationic protein showed similar physiologic effects, possibly via direct activation of G-protein by basic peptides (Mousli et al., 1989; Mousli et al., 1990). SP^{4-11} was the most potent bronchoconstrictor fragment. The presence of proline at position 4 may account for this as presence of proline can modify the conformation of the carboxy-terminal sequence (Cascieri et al., 1992), to optimize its interaction within the binding pocket of the NK2 receptor. However, further addition of one to three amino acids (lysine, proline and arginine) may provide additional binding interactions or modify the conformation of carboxy-terminal to favour binding to NK1 receptors whose activation leads to less bronchospastic effect than activation of NK2 receptors.

The selective NK1 receptor antagonists CP 96,345 or CP 99,994 and the selective NK2 receptor antagonist SR 48,968 did not change the baseline values of R_L and E_L indicating that their effects are not via nonspecific effects on baseline airway calibre or airway smooth muscle contractility. CP 96,345 or CP 99,994 greatly reduced responses to SP and SP fragments. By contrast, SR 48,968, in a dose that blocked responses to NKA and the selective NK2 receptor agonist [β-Ala^8]-NKA 4-10, significantly reduced responses to only SP$^{4-11}$. These findings suggest that SP and its C-terminal fragments induce bronchoconstriction mainly via NK1 receptors. As a selective NK2 receptor antagonist reduced bronchospasm induced by SP$^{4-11}$, the greater bronchoconstrictor activity of SP$^{4-11}$ may, at least in part, be explained by its effects being mediated via NK2 receptors. A combination of NK1 and NK2 receptor antagonists caused greater rightward shift of the dose-response curves to SP and SP fragments suggesting that bronchoconstriction induced by SP or SP fragments is mediated via both NK1 and NK2 receptors in guinea-pig. These findings are supported by those of Bertrand et al. (1993) and Foulon et al. (1993) who showed that in guinea-pigs both the NK1 and NK2 receptors must be involved in bronchospasm induced by these agonists. Ballati et al. (1992) claimed that capsaicin's bronchospastic effects were reduced by capsaicin-induced release of TK that had stimulatory actions on NK2 receptors in sympathetic ganglia. This may explain the ineffectiveness of selective NK2 receptor antagonist on bronchospasm induced by these peptides while in combination with selective NK1 receptor antagonists it induced significance decrease in this response. Studies on the effects of septide, an analogue of SP C-terminal which was belived to be a specific NK1 receptor agonist, have shown the existence of a distinct new NKR named "septide -sensitive" receptor.[32] It has been suggested that NK1 receptor agonists and septide act on different sites of the receptor protein which leads to distinct conformational changes induced by these peptides (Pradier et al., 1994). Mutational studies have shown that there may be conformational compatibility between each antagonist and NKR so that binding to one receptor may lead to specific changes which are different from changes which occure when both receptors are occupied (Huang et al., 1994a). It is possible that both NK1 and NK2 receptors are located on the same cell or NK1 and NK2 receptor antagonists act on different sites of the same protein. This may explain the greater effect of combination of NK1 and NK2 receptor antagonist in blocking bronchoconstriction induced by SP and its fragments.

In conclusion, SP^{5-11}, SP^{3-11}, SP^{2-11} and SP^{6-11} induce bronchoconstriction mainly via NK1 receptors; SP^{4-11} acts via NK1 and NK2 receptors. N-terminal fragments of SP were devoid of bronchospastic activity.

CHAPTER 6

MECHANISMS INVOLVED IN BRONCHOPULMONARY RESPONSES TO CAPSAICIN

A version of this paper has been submitted to Pulmonary Pharmacology for publication.

6.1. INTRODUCTION

In many tissues, capsaicin selectively stimulates a sub-population of sensory parasympathetic and sympathetic afferent nerves to release neuropeptides (Saria et al., 1988; Manzini et al., 1989c; O'Neill, 1991).Thus, in perfused guinea-pig lungs, *in vitro*, low concentrations of capsaicin induced the release of SP, NKA and CGRP (Saria et al., 1984; Lundberg & Saria, 1988; Martling et al., 1988) and, *in vivo*, non-selective or selective NKR antagonists reduced or abolished capsaicin-induced bronchospasm (Ballati et al., 1992; Bertrand et al., 1993). Capsaicin acts on "vanilloid" receptors, non-selective cation channels, on the nerve terminal (Maggi & Meli, 1988; O'Neill, 1991). Stimulation of vanilloid receptors leads to the entry of Na^+ and Ca^{2+} ions into the sensory nerve terminals, to their depolarization and to the release of neuropeptides (Dray, 1992; Lundberg et al., 1992). Whether depolarization of these sensory endings evokes central or axonal reflex-mediated effects is controversial (Biggs & Goel, 1985; Maggi & Meli, 1988; Kroll et al., 1990). Most investigators showed that axonal reflexes had no role in the capsaicin-induced release of neuropeptides (Molnar et al., 1969; Biggs & Goel, 1985). By contrast, there are conflicting views on the role of centrally mediated-reflexes in capsaicin's pulmonary effects, *in vivo*. Some have found pharmacologic evidence for central reflex effects (Delpierre et al., 1981; Ballati et al., 1993), others have found none (Szolcsanyi, 1983; Kroll et al., 1990). However, there is general agreement that at least part of capsaicin's bronchospastic effects are mediated by its ability to release neuropeptides from sensory nerve endings. Thus, NK2 receptor antagonists, or the destruction of sensory nerve endings by pretreatment with large amounts of capsaicin, greatly reduces or prevents capsaicin-induced bronchospasm (Karlsson & Persson, 1985; Saria et al., 1985). Although, there is good evidence for NK1 receptors mediating bronchospasm (Longmore et al., 1994), surprisingly, NK1 receptor antagonists alone have little effect on capsaicin-induced bronchospasm (Maggi, 1990; Lilly et al., 1994). However, both NK1 and NK2 receptor blockade is required to abolish capsaicin's pulmonary effects (Maggi, 1990; Ballati et al., 1992; Bertrand et al., 1993).

CGRP is co-released by capsaicin with SP and NKA; its role in capsaicin's bronchospastic effects is unclear. Although CGRP has no effect on guinea-pig airway smooth muscle, *in vitro* (Martling et al., 1988; Luts et al., 1990; Parsons et al., 1992), in many tissues, there is good evidence that CGRP has neuromodulatory as well as direct effects (Cadieux et al., 1990; Cadieux & Lanoue, 1990). We characterized capsaicin-induced bronchospasm in anesthetized guinea-pigs using a specific monoclonal antibody against SP and selective NKR agonists and antagonists. Also, we determined the effects of hCGRP and a CGRP antagonist ($CGRP^{8-37}$) on capsaicin-induced pulmonary responses.

6.2. METHODS AND MATERIALS

6.2.1. Animals

SPF-quality, female Hartley-strain guinea-pigs (weight range: 350-450 g) were obtained from Charles River Inc., St. Constant, Québec. They were transported to Edmonton in filter-top boxes and housed in laminar flow units (Bioclean™, Hazleton) on grids over trays of rock salt and fed guinea-pig chow supplemented with apples. Water was allowed *ad lib.* All experimental procedures were reviewed and approved by the Health Sciences Animal Welfare Committeee, University of Alberta.

6.2.2. Methods

6.2.2.1. Pulmonary measurements

Guinea-pigs were anesthetized with sodium pentobarbital (40-50 mg kg^{-1}, ip) with additional doses (5-10 mg kg^{-1}, iv) as required. Their tracheas were cannulated about 1 cm caudal to the larynx (PE240, 3 cm) and artificial respiration was applied (tidal volume = 9 ml kg^{-1}, pump speed = 20 strokes min^{-1}) with a rodent ventilator (Ugo Basile, Varese, Italy). A jugular vein was cannulated (PE50, 20 cm, attached to a 23G1 needle, total dead space = 0.06 ml) for giving drugs iv. All animals received succinylcholine (0.03 mg kg^{-1}, iv) to prevent spontaneous respiratory movements that might interfere with measurements. Air flow was measured via a pneumotach (Fleisch 0000, Validyne MP45 differential pressure transducer) and intratracheal pressure relative to atmosphere via a pressure transducer (Validyne MP45). Signals were digitized and processed to yield breath-by-breath values of R_L and E_L, using a computerized system. In some experiments, arterial blood pressure was monitored via a catheter (PE50) placed in a carotid artery (Statham P23Dd pressure transducer, HP 8805B carrier amplifier, HP 7702B chart recorder). Dose- (ascending order, responses allowed to return to baseline before injection of the next dose) response (as absolute values or as percent change from baseline values of R_L or E_L) curves were established to capsaicin, SP, NKA, [Sar^9,Met(O$_2$)^{11}]-SP and [β-Ala^8]-NKA$^{4-10}$ in separate control and test groups of animals. Antagonists, autonomic blockers, hCGRP$^{8-37}$ and control vehicle were given (iv) 15 min before agonists. In one set of experiments, α-SP MAb was given (iv) 30 min before capsaicin; in another, it was given 24 h (ip) and 30 min (iv) before animals were used for experiments. Bilateral vagotomy was performed midcervically. hCGRP was given alone, or immediately before injection of SP, NKA, or capsaicin.

6.2.3. Materials

Drugs used were: substance P, neurokinin A and human calcitonin-gene-related peptide (hCGRP) (Biochem Pharma Inc., Montréal, PQ); [Sar^9,Met(O$_2$)^{11}]-SP, [β-Ala^8]-NKA$^{4-10}$, and human calcitonin gene-related peptide$^{8-37}$ (hCGRP$^{8-37}$) (Peninsula Laboratories Inc., Belmont, CA); atropine sulphate (BDH, Toronto, ON); mecamylamine hydrochloride (gift of Merck, Sharp & Dohme Research Lab., Rahway, NJ); nadolol (gift of Bristol-Myers Squibb Canada Inc., Montréal, PQ); pelargonic acid vanillylamide (synthetic capsaicin) (Fluka AG, CH-9470 Buchs, Switzerland); sodium pentobarbital (Euthanyl,™ M.T.C Pharmaceuticals, Markham, ON); succinylcholine chloride (Sigma, St. Louis, MO). CP 96,345 was a gift of Pfizer Inc., Groton, CO; SR 48,968 (gift of Sanofi Récherche, Montpellier, France). α-SP MAb was obtained from the rat/mouse heterohybridoma NC1/34 (Jafarian et al., 1995).

6.2.4. Statistical analyses

Points comprising dose-response curves were plotted as mean ± SEM. Data were analyzed using Sigmastat™, Mann-Whitney rank sum tests, Student's *t* tests. One- and two-way ANOVA were used to compare differences among responses. Regression analyses were used to determine linearity and confidence limits. Significance was assumed at the 5% level.

6.3. RESULTS

6.3.1. Bronchopulmonary responses to capsaicin and effects of autonomic blockers

Capsaicin (4.0-30.0 μg kg^{-1}, iv) induced dose-dependent changes in R_L and E_L (Fig. 6.1). As responses to the largest doses of capsaicin were slow to return to baseline values and showed evidence of a cumulative effect of the drug, separate groups of control and test animals were used.

Baseline values of R_L and E_L were similar in guinea-pigs pretreated with the muscarinic blocker atropine (0.5 mg kg^{-1}, iv; $p > 0.05$, $n = 4$) or bilaterally vagotomized ($p > 0.05$, $n = 3$) and untreated controls. Dose-response curves to capsaicin in atropinized or vagotomized animals were similar to those in untreated controls (Fig. 6.1; $p > 0.05$, $n = 4$ or 3). By contrast, although the ganglionic blocker mecamylamine (5 mg kg^{-1}, iv) did not alter baseline values of R_L and E_L compared to untreated controls, it significantly increased bronchopulmonary responses to capsaicin (Fig. 6.1; $p < 0.05$, $n = 3$). The non-specific β-receptor blocker nadolol (1.0 mg kg^{-1}, iv) significantly increased baseline values of R_L and E_L (R_L: 0.189 ± 0.004 to 0.254 ± 0.023, $p < 0.05$, $n = 6$; E_L: 1.693 ± 0.044 to 2.232 ± 0.17, $p < 0.05$, $n = 6$). Nadolol significantly increased responses to capsaicin ($n = 3$, $p < 0.05$) (Fig. 6.1). After correcting for changes in baseline, analysis of responses to capsaicin in guinea-pigs pretreated with nadolol indicated that the responses were significantly greater ($p < 0.05$) than those in control or mecamylamine-treated animals (Fig. 6.2). Combinations of mecamylamine and nadolol increased bronchopulmonary responses to capsaicin more than either drug alone (Figs. 6.1 - 6.2). After correcting for changes in baseline, two-way ANOVA indicated that the drugs' effects were additive. Findings for E_L (not shown) were similar to those for R_L.

6.3.2. Effects of α-SP MAb on bronchopulmonary responses to capsaicin

In doses that significantly inhibited responses to SP or NKA, (Jafrian et al., 1995) α-SP MAb (iv), did not alter baseline values of R_L and E_L (R_L: before = 0.26 ± 0.03; after = 0.27 ± 0.03, $p > 0.05$, $n = 4$; E_L: before = 2.75 ± 0.015, after = 2.67 ± 0.42, $p > 0.05$, $n = 4$). Also, baseline values of R_L and E_L in the α-SP MAb-treated and vehicle-treated control groups were similar (R_L: test = 0.26 ± 0.03, control = 0.27 ± 0.03, $p > 0.05$, $n = 4$; E_L test = 2.75 ± 0.43, control = 2.68 ± 0.41, $p > 0.05$, $n = 4$). Given only iv, α-SP MAb did not alter the changes in R_L and E_L induced by capsaicin (Fig. 6.3). By contrast, α-SP MAb given 24 h (ip) and 30 min (iv) before capsaicin, significantly reduced capsaicin-induced changes in R_L and E_L ($n = 3$, $p < 0.05$) (Fig. 6.3). An "irrelevant" rat IgG antibody given in similar doses 24 h (ip) and 30 min (iv) before capsaicin did not alter bronchopulmonary responses to capsaicin (data not shown).

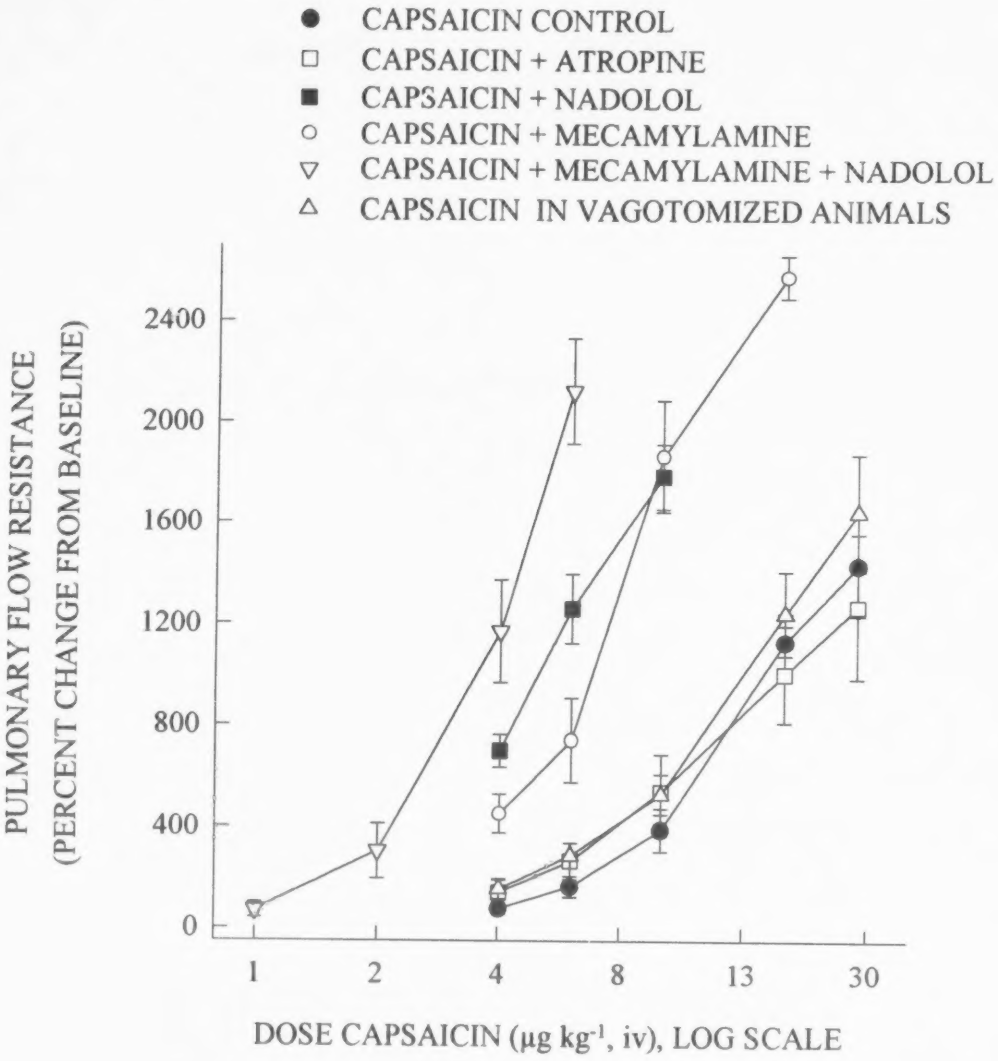


Fig. 6.1

The effects of autonomic blockers on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Results as percent change from baseline values are mean ± SEM of 3-6 experiments.

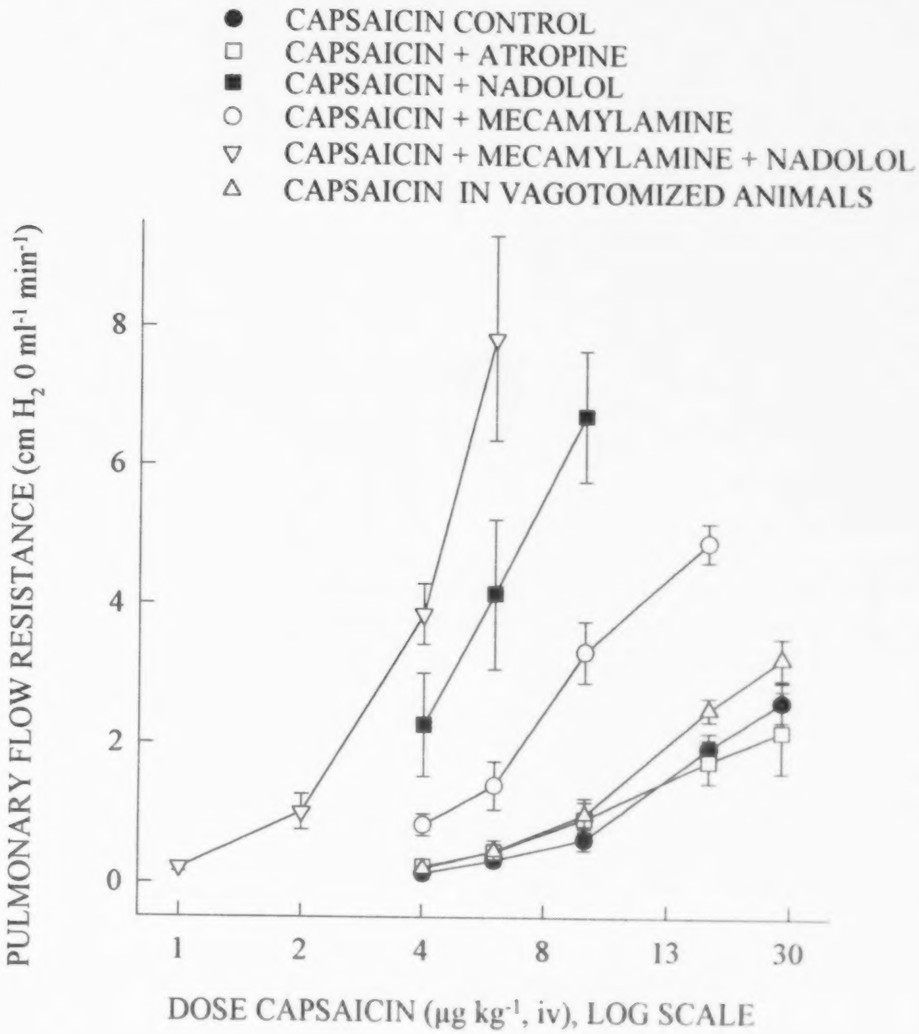


Fig. 6.2

The effects of autonomic blockers on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Results as increase in R_L are mean ± SEM of 3-6 experiments.

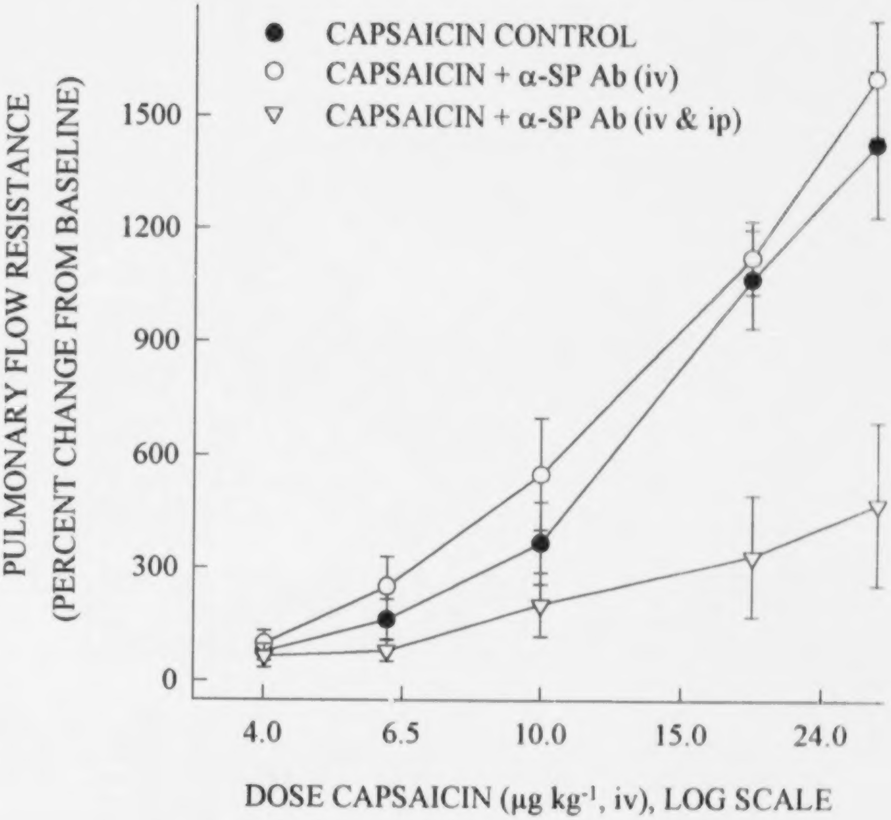


Fig. 6.3

The effects of α-SP MAb on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Ab was given either 30 min (iv) or 24 h (ip) and 30 min (iv) beforehand. Results, expressed as percent change from baseline values, are mean ± SEM of 3-6 experiments.

6.3.3. Bronchopulmonary responses induced by SP, NKA, [Sar^9,Met(O$_2$)^{11}]-SP, [β-Ala^8]-NKA and capsaicin

SP (1.0-8.0 μg kg^{-1}), NKA (0.1-1.0 μg kg^{-1}), [Sar^9,Met(O$_2$)^{11}]-SP (0.2-1.0 μg kg^{-1}), [β-Ala^8]-NKA$^{4-10}$ (1.0-6.0 μg kg^{-1}) and capsaicin (5.0-30.0 μg kg^{-1}) (all iv) induced dose-dependent increases in R_L and E_L in the control groups (Figs. 6.4A & B, 6.5A & B, 6.6A & B and 6.7). The onset of SP-, [Sar^9,Met(O$_2$)^{11}]-SP-, NKA and [β-Ala^8]-NKA$^{4-10}$-induced bronchospasm and recovery to baseline were slower than those of capsaicin (Table 6.1).

6.3.4. Effects of NKR antagonists on bronchopulmonary responses induced by SP, NKA, [Sar^9,Met(O$_2$)^{11}]-SP, [β-Ala^8]-NKA and capsaicin

The NK1 receptor antagonist CP 96,345 (1.0-3.0 mg kg^{-1}) did not alter baseline values of R_L and E_L, but reduced responses to SP and blocked responses to the selective NK1-receptor agonist, [Sar^9,Met(O$_2$)^{11}]-SP (Figs. 6.4A & B, 6.5A & B, 6.6A & B). However, CP 96,345 did not reduce or block responses to capsaicin (Fig. 6.7). Moreover, in some guinea-pigs (2 of 7) CP 96,345 significantly ($p < 0.05$) *increased* bronchospastic responses to capsaicin (Fig. 6.8).

The NK2 receptor antagonist SR 48,968 (0.1-1.0 mg kg^{-1}, iv) had no effect on baseline values of R_L and E_L, but blocked responses to NKA and [β-Ala^8]-NKA$^{4-10}$, and greatly reduced, but did not abolish, pulmonary responses to capsaicin (Figs. 6.4A & B, 6.5A & B, 6.6A & B and 6.7). A combination of the NK1 and NK2 receptor antagonists eliminated bronchopulmonary responses to capsaicin (Fig. 6.7).

TABLE 6.1

Time (s) required to achieve maximal bronchospasm (peak response) after injection of agonists iv (t max) and the half life ($t_{1/2}$) of the peak response. Data are expressed as mean ± SEM of 4-6 experiments.

AGONIST	t max (s)	$t_{1/2}$, PEAK RESPONSE (s)
Capsaicin	10.5 ± 0.9	12.0 ± 0.3
[Sar^9,Met(O$_2$)^{11}]-SP	20.5 ± 4.5	18.1 ± 1.8
Substance P	13.8 ± 1.2	20.1 ± 0.6
Neurokinin A	75.0 ± 10.2	55.6 ± 3.6
[β-Ala^8]-NKA$^{4-10}$	67.4 ± 8.8	67.3 ± 4.9

6.3.5. Effects of hCGRP and hCGRP$^{8-37}$ on bronchopulmonary responses to SP, NKA or capsaicin

In doses that significantly decreased mean arterial blood pressure, hCGRP (iv) did not alter baseline values of R_L and E_L or the changes in R_L and E_L induced by SP, NKA or capsaicin (n = 4, $p > 0.05$) (Figs. 6.9 - 6.10). By contrast, hCGRP,$^{8-37}$ a CGRP antagonist (Bartho et al, 1991; Maggi et al., 1991d), in doses that did not alter baseline values of R_L and E_L but reduced the depressor effects of hCGRP (Fig. 6.11), significantly ($p < 0.05$) increased pulmonary responses induced by capsaicin (Fig. 6.12).

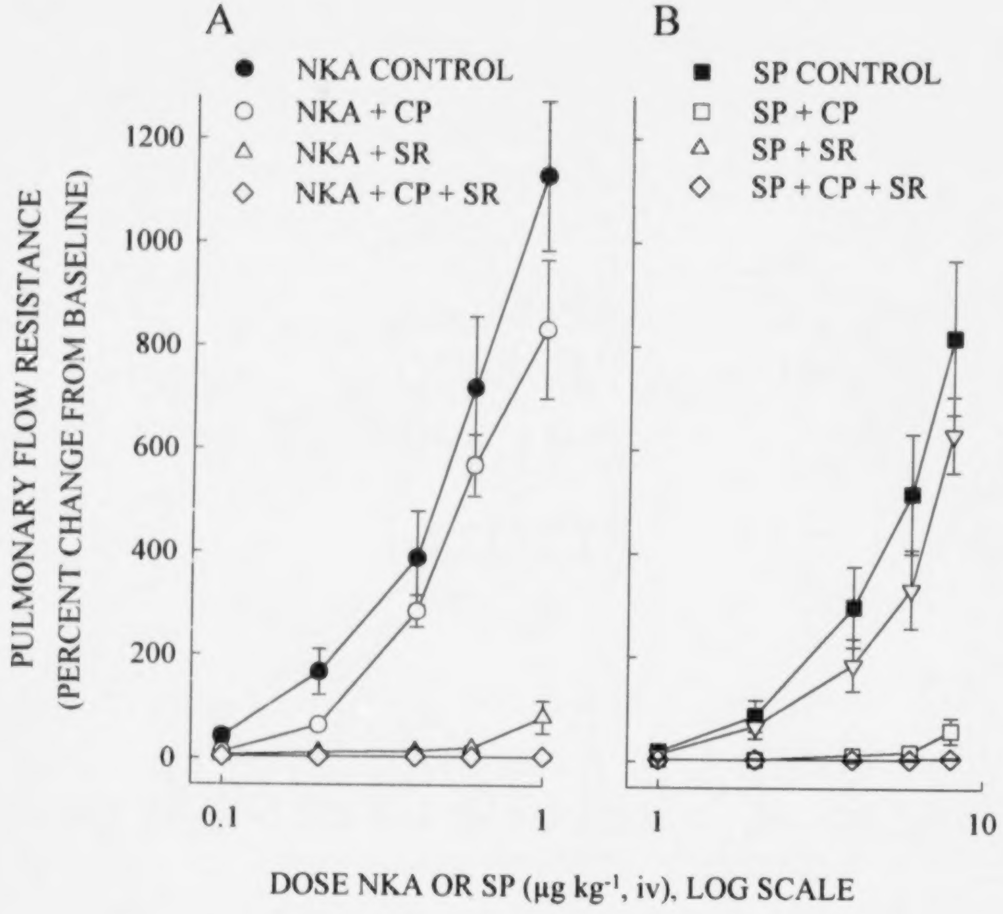


Fig. 6.4

The effects of CP 96,345 (selective NK1 receptor antagonist) and SR 48,968 (selective NK2 receptor antagonist) on changes in pulmonary flow resistance in response to various doses of NKA (A) and SP (B) (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4-6 experiments.

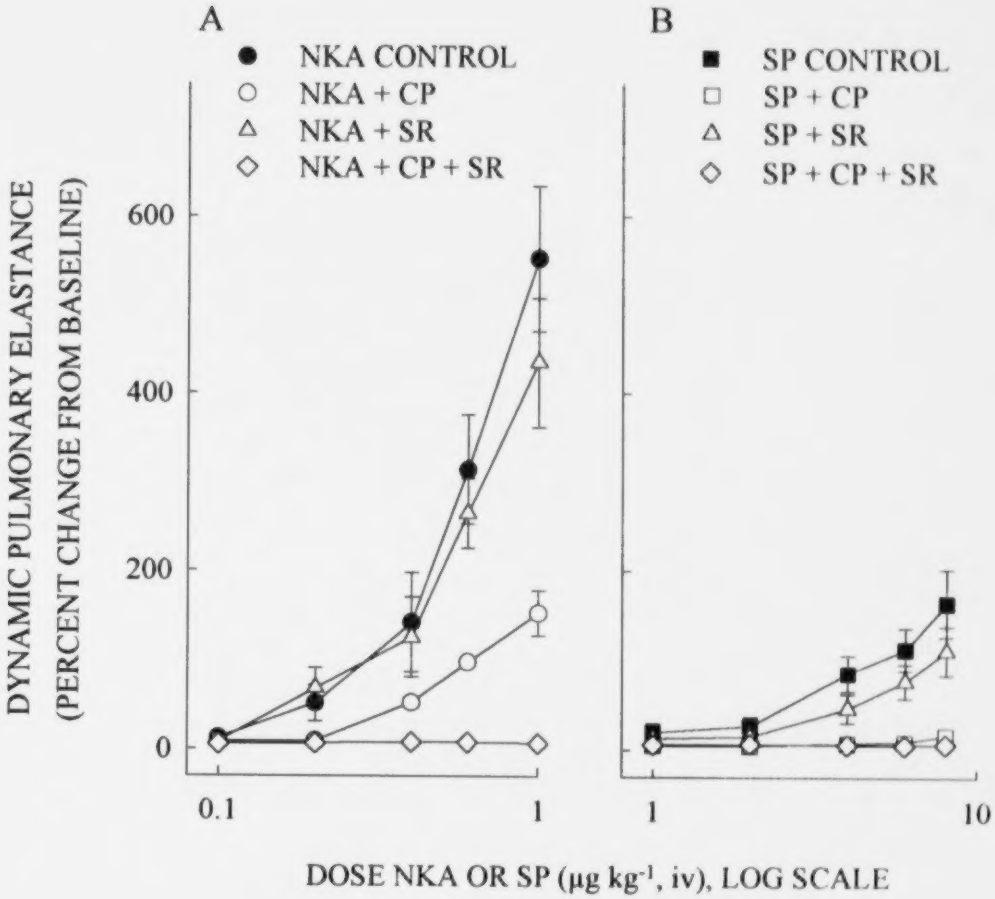


Fig. 6.5

The effects of CP 96,345 (selective NK1 receptor antagonist) and SR 48,968 (selective NK2 receptor antagonist) on changes in dynamic pulmonary elastance in response to various doses of NKA (A) and SP (B) (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4-6 experiments.

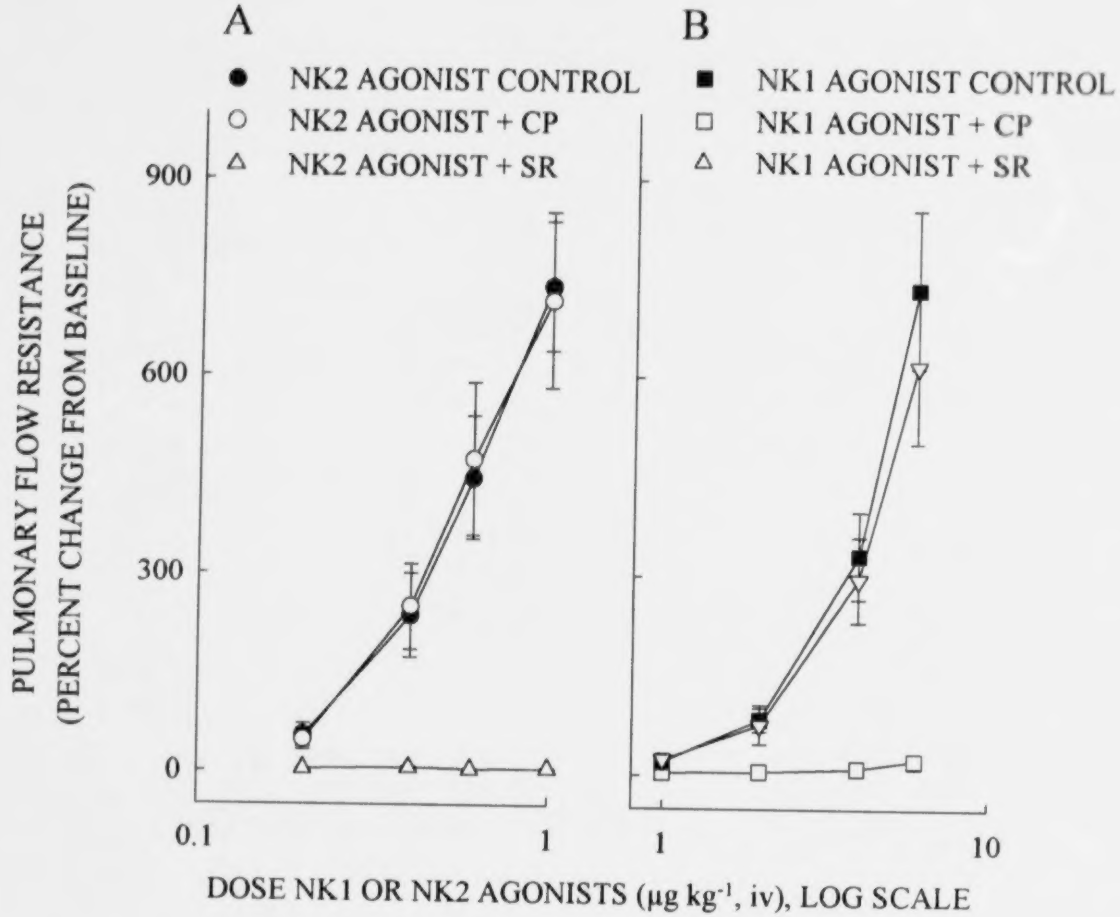


Fig. 6.6

The effects of CP 96,345 (selective NK1 receptor antagonist) and SR 48,968 (selective NK2 receptor antagonist) on changes in pulmonary flow resistance in response to various doses of selective NK2 (A) and NK1 (B) receptor agonists (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4 experiments.

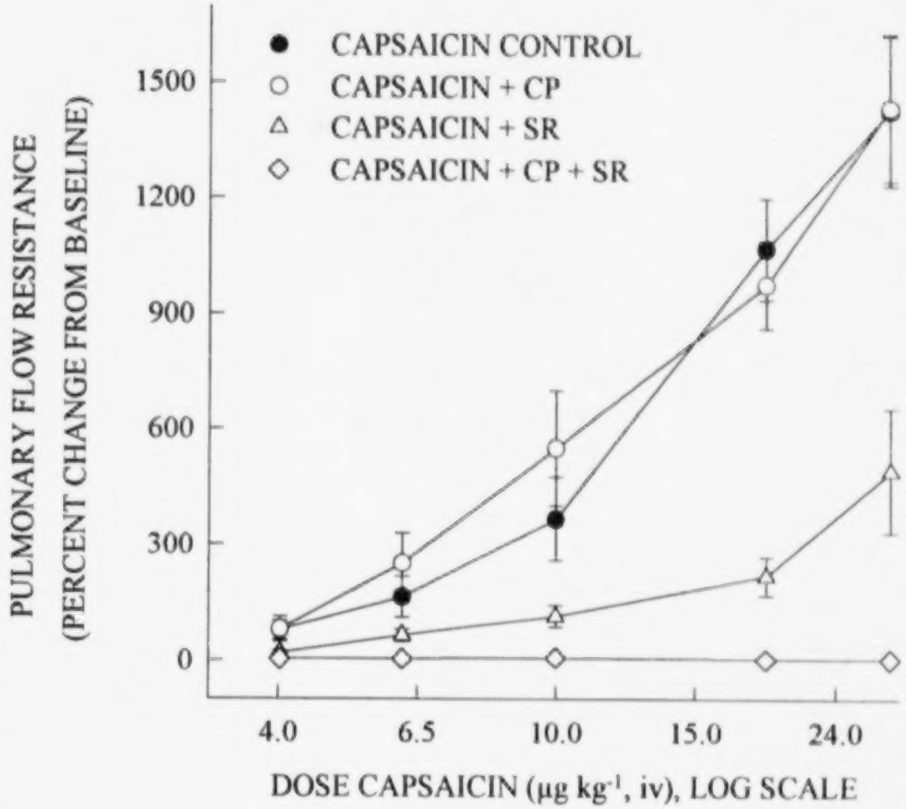


Fig. 6.7

The effects of CP 96,345 (selective NK1 receptor antagonist) and SR 48,968 (selective NK2 receptor antagonist) on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4-6 experiments.

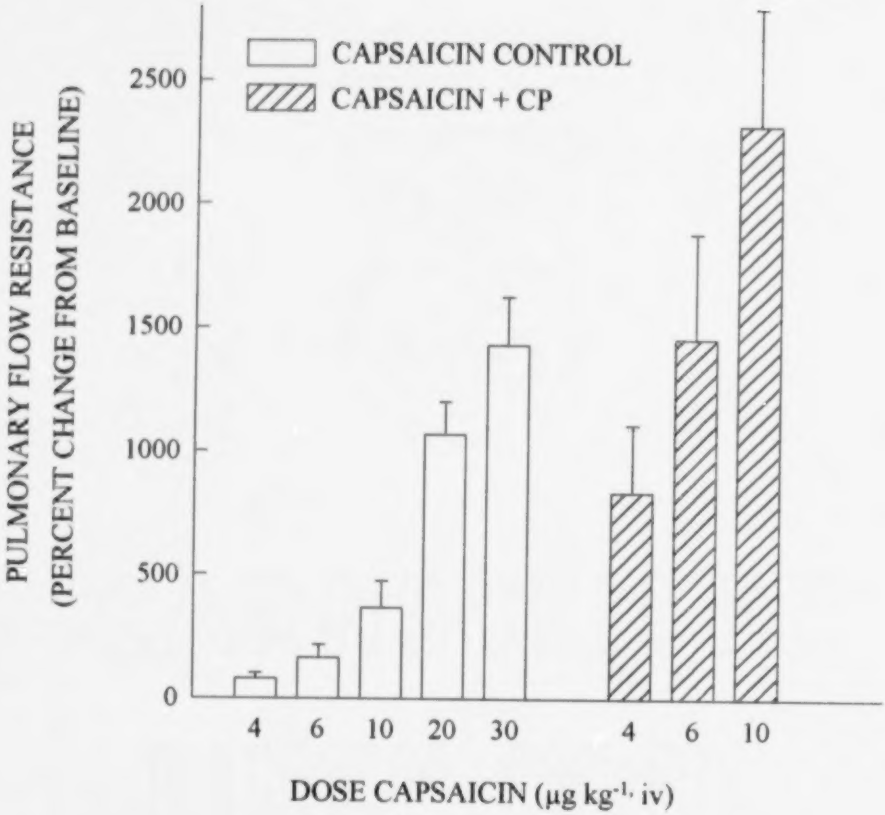


Fig. 6.8

The effects of CP 96,345 (selective NK1 receptor antagonist) on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 2 experiments.

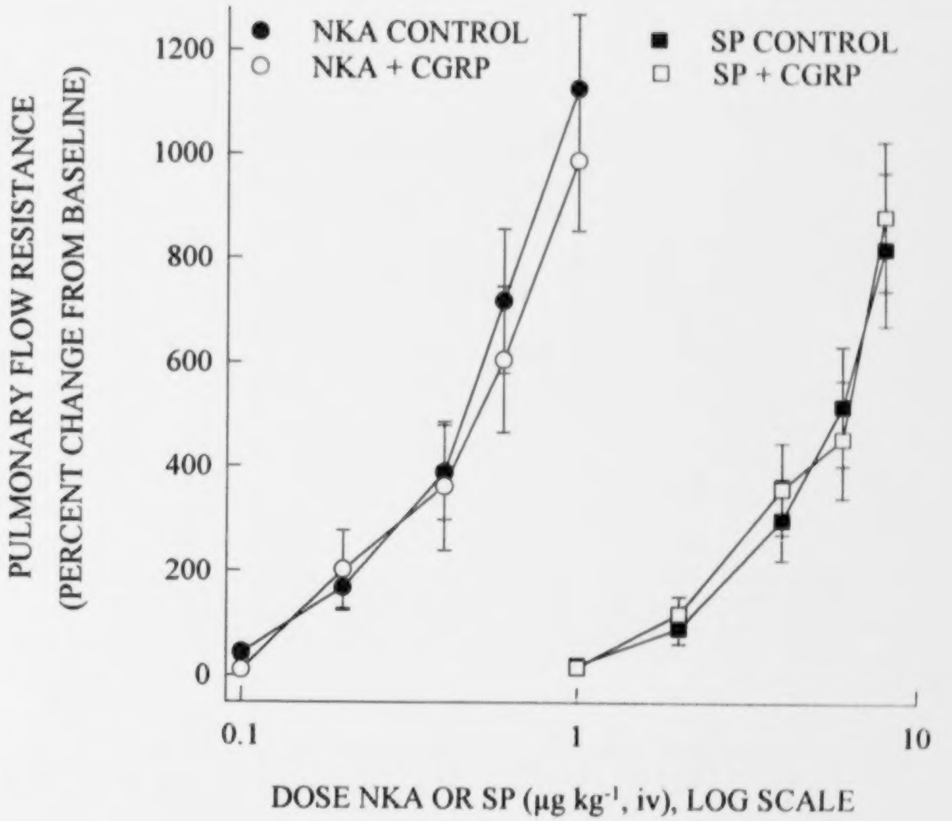


Fig. 6.9

The effects of CGRP on changes in pulmonary flow resistance in response to various doses of SP and NKA (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4-6 experiments.

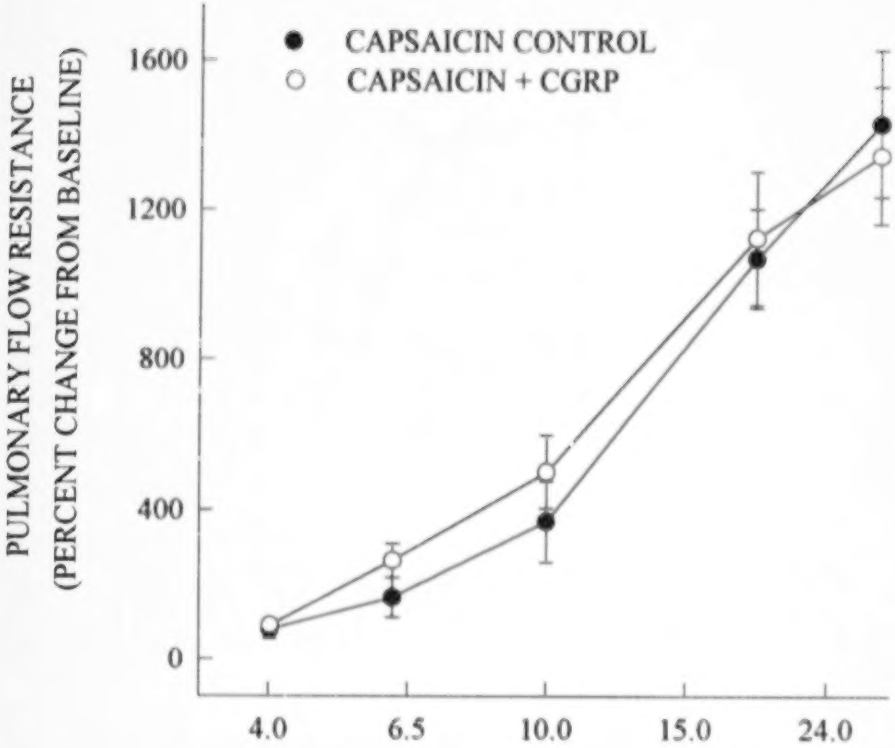


Fig. 6.10

The effects of CGRP on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4 or 6 experiments.

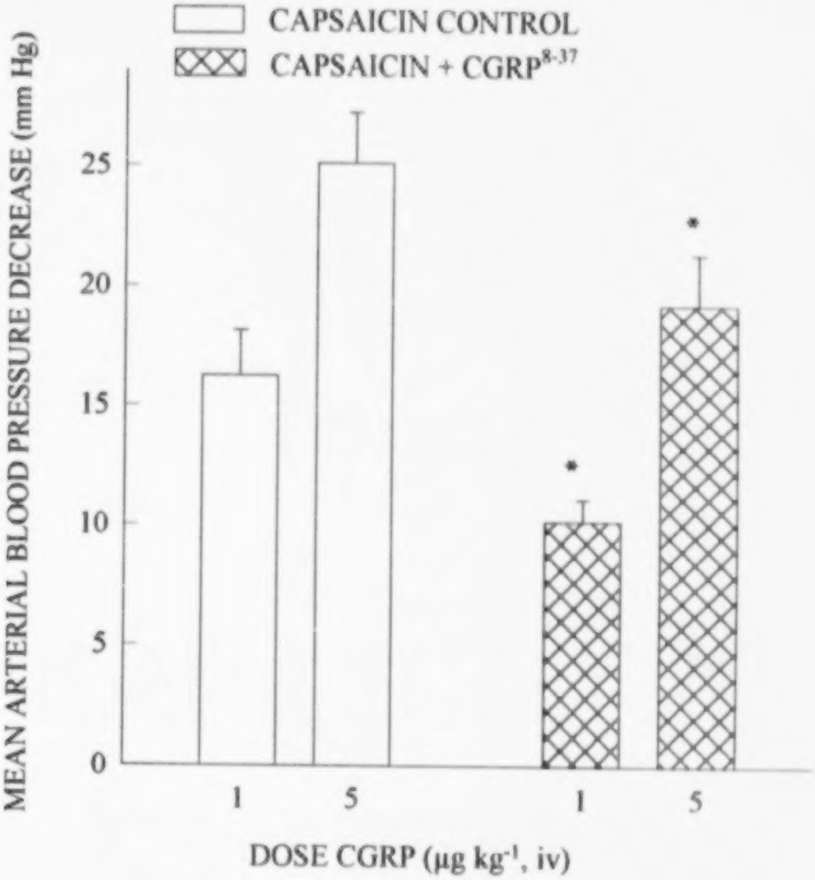


Fig. 6.11

The effects of CGRP$^{8-37}$ on the depressor effects of CGRP (iv). Results, expressed as change in mean arterial blood pressure (mm Hg), are mean ± SEM of 4 experiments.

(* $p < 0.05$)

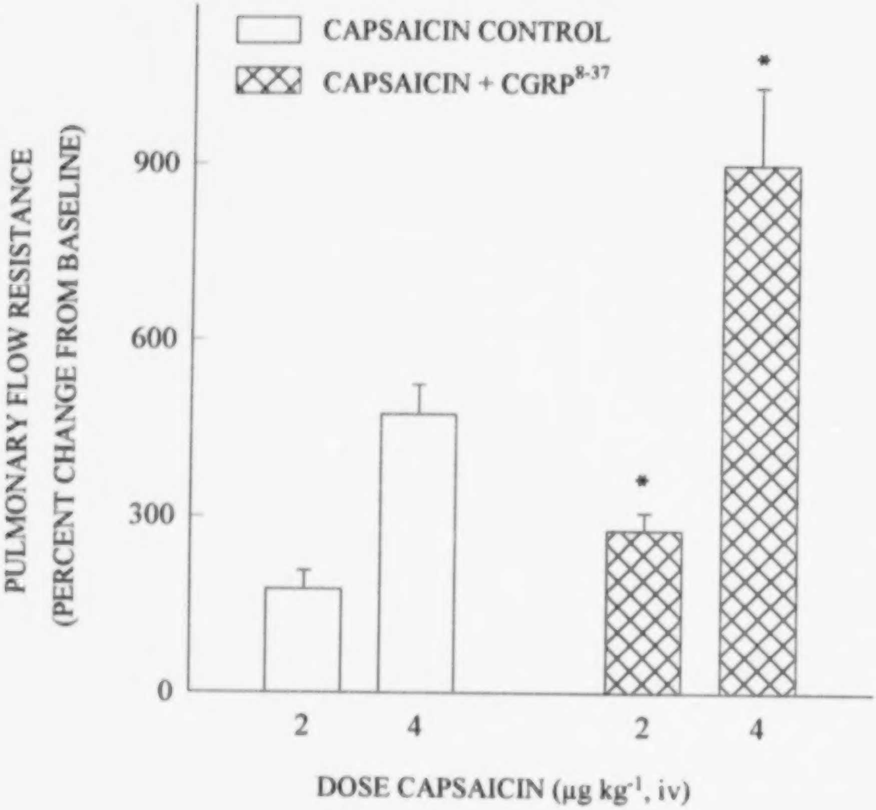


Fig. 6.12

The effects of CGRP$^{8-37}$ on changes in pulmonary flow resistance in response to various doses of capsaicin (iv). Results, expressed as percent change from baseline values, are mean ± SEM of 4 experiments.

(* $p < 0.05$)

6.4. DISCUSSION

SPF-quality guinea-pigs, shipped in filter top boxes and maintained in laminar flow units that supplied HEPA-flitered air were used in these experiments. Guinea-pigs were maintained on grids over trays of rock salt and routine, periodic histologic examinations of animals' lungs revealed no pathologic findings. Thus, it is unlikely that responses to tachykinins and capsaicin were enhanced or modified by the presence of infections or environmental factors such as ammonia (Fryer & Jacoby, 1991; McDonald et al., 1991; McDonald, 1992).

Capsaicin may induce bronchospasm via direct effects on afferent nerve endings that release SP and NKA, via axonal reflexes that release these tachykinins, via centrally mediated reflexes whose efferent arc comprises the parasympathetic nervous system, or combinations of these effects (Maggi & Meli, 1984). Some workers (Molnar et al., 1962; Szolcsanyi, 1983; Maggi & Meli, 1984; Biggs & Goel, 1985) concluded that there was no *central* cholinergic reflex component to capsaicin-induced bronchospasm in guinea-pigs. Others (Delpierre et al., 1981; Kroll et al., 1990; Ballati et al., 1992) concluded the opposite. Ballati et al. (1992) who studied the effects of single doses of capsaicin, selective NK1 and NK2 receptor agonists and acetylcholine in animals treated with atropine or hexamethonium, the non-specific β-blocker propranolol, or pharmacologically sympathectomized with guanethidine, suggested that part of capsaicin's effects involved centrally mediated cholinergic reflexes and that part resulted from the capsaicin-induced release of endogenous tachykinins acting on airway smooth muscle NK2 receptors. The latter component was modulated via tachykinin-mediated stimulation of NK2 receptors situated on sympathetic ganglia. They concluded that capsaicin released tachykinins that stimulated sympathetic ganglia, released sympathomimetics and thus antagonized its own indirect bronchospastic effects. We found no evidence for cholinergically mediated effects of capsaicin. In agreement with earlier findings (Biggs & Goel, 1985), in the experiments reported here, neither atropine, nor bilateral vagotomy, nor mecamylamine reduced bronchospastic responses to capsaicin. Thus, we have no evidence implicating central or axonal reflex-mediated parasympathetic effects in capsaicin-induced bronchospasm.

Pretreatment of animals with the ganglionic blocker mecamylamine or the non-specific β-receptor blocker nadolol enhanced responses to capsaicin and shifted dose-response curves significantly to the left. Similar findings with a single dose of capsaicin were reported by Ballati et al. (1992) with propranolol and hexamethonium. In the experiments reported here, nadolol increased baseline values of R_L and E_L, but mecamylamine was without effect on these parameters. The increase in baseline after giving nadolol could account, in part, for the enhancement of bronchopulmonary responses to capsaicin. These findings are in agreement with those of others (Advenier et al., 1983; Stewart et al., 1984) using the ganglionic blocker hexamethonium and histamine or leukotriene D_4 as agonists. Paradoxically, combinations of nadolol and mecamylamine showed an additive enhancement of capsaicin-induced bronchospasm. Mecamylamine's and part of nadolol's effects could be attributed to block of sympathetic neurotransmission. Thus, in our experimental situation, the airways have sympathetic tone. "Nonspecific" effects of nadolol could account for additive effect of combinations of nadolol and mecamylamine and the increase in baseline values of R_L and E_L. RS-, R- and

S-propranolol and the cardioselective β_1-blocker, practolol, all enhance the bronchoconstrictor effects of histamine (Ney, 1983). Although, we did not specifically design experiments to demonstrate that capsaicin increases sympathetic activity via NK2-receptor mediated effects at sympathetic ganglia, our findings with mecamylamine and nadolol suggest that this effect was not a feature of our experiments. Moreover, the findings with CP 96,345, the selective NK1 receptor antagonist, in which we noted marked potentiation of responses to capsaicin in two of seven animals, suggest involvement of NK1 not NK2 receptors. Our findings are in agreement with those of others (Foulon et al., 1993) who showed that NK1 receptors, but not NK2, receptors were involved in neurokinin-induced depolarization of tonic neurons in the guinea-pig.

α-SP MAb, in doses that significantly inhibited SP- or NKA-induced bronchospasm (Jafarian et al., 1995), when given ip and iv, decreased capsaicin-induced changes in R_L and E_L. The inability of α-SP MAb given only iv (30 min beforehand) to reach the sites of capsaicin-induced neurokinin release may account for its lack of effect against capsaicin as it was effective when given ip and iv. An irrelevant rat monoclonal IgG antibody was ineffective confirming the specificity of the effect observed. The findings with this specific α-SP MAb confirm that the endogenous release of the neurokinins, SP and NKA, mediates bronchoconstriction induced by capsaicin.

The onset of SP-, [Sar^9,Met(O$_2$)^{11}]-SP-, NKA and [β-Ala^8]-NKA$^{4\text{-}10}$-induced bronchospasm and recovery to baseline were slower than those of capsaicin. It is likely that endogenously released SP and NKA induce more rapid bronchoconstriction as the distance between the site of release and the receptor is small (McDonald, 1994) compared with exogenously applied SP and NKA.

The selective NK1 receptor antagonist CP 96,345, in a dose that reduced responses to SP and blocked responses to the selective NK1 receptor agonist [Sar^9,Met(O$_2$)^{11}]-SP, had no effect on bronchopulmonary responses to capsaicin in five of seven guinea-pigs. However, the selective NK2 receptor antagonist SR 48,968, in a dose that blocked responses to NKA and the selective NK2 receptor agonist [β-Ala^8]-NKA$^{4-10}$, greatly reduced bronchopulmonary responses to capsaicin but did not eliminate them. A combination of NK1 and NK2 receptor antagonists was necessary to eliminate them. These findings agree with those of others (Bertrand et al., 1993; Foulon et al. 1993) and, together with those using α-SP MAb, show that endogenous release of the neurokinins, SP and NKA, mediate bronchoconstriction induced by capsaicin via NK1 and NK2 receptors; latter dominate capsaicin's pulmonary effects. The NK1 antagonist induced augmentation of capsaicin-induced bronchospasm seen in some animals suggests that there may be a presynaptic NK1 receptor-mediated inhibitory system that decreases TK release from capsaicin sensitive afferent nerves. As NKA and [β-Ala^8]-NKA$^{4-10}$ were more effective than SP at inducing changes in E_L, NK2 receptors appear to be more abundant in the distensible airways (Joos & Pauwels, 1990).

There are conflicting findings regarding the effects of CGRP on airways. Thus, Palmer et al. (1987a) concluded that CGRP was a potent constrictor of human airways. By contrast, Martling et al. (1990) concluded that CGRP had a relaxant effect on precontracted pig bronchi. Other studies (Cadieux et al., 1990; Cadieux & Lanoue, 1990; Kroll et al., 1990) showed that CGRP lacked contractile activity in the airways but reduced responses induced by bronchoconstrictor agents. Our findings revealed that hCGRP in doses that significantly decreased mean arterial blood pressure, had no effect on changes in R_L and E_L induced by SP, NKA or capsaicin. These findings are in agreement with those of Martling et al. (1988) who showed neither rat nor human CGRP induced bronchoconstriction, or inhibition of NKA-induced bronchospasm. As suggested by others (Martling et al., 1990; Mimeault et al., 1991; Evangilista et al., 1992; Giuliani et al., 1992; Bartho et al., 1993), the presence of multiple CGRP receptor subtypes could account for these differences. It is noteworthy that the hCGRP antagonist, in a dose that reduced the depressor effect of hCGRP, *increased* bronchopulmonary responses to capsaicin. $hCGRP^{8-37}$ antagonizes the effects of CGRP in various tissues (Bartho et al., 1991). The ability of $hCGRP^{8-37}$ to increase capsaicin-induced bronchoconstriction may be due to its blocking effect on CGRP released from capsaicin sensitive C-fiber afferents. CGRP is colocalized and coreleased with SP and NKA (Martling et al., 1988; Lundbrg et al., 1992). Although we were unable to demonstrate that hCGRP blocked the effects of bronchoconstrictor agonists, these findings suggest that the capsaicin-induced release of CGRP may modulate bronchopulmonary responses induced by the release of endogenous SP and NKA.

In conclusion, the endogenous release of the neurokinins, SP and NKA, acting on NK2, and to a lesser extent NK1 receptors, mediates bronchoconstriction induced by capsaicin. No central or local cholinergic reflexes are involved in these responses. Bronchospastic responses to capsaicin mediated via the release of endogenous neurokinins are modulated by the concomitant release of endogenous CGRP.

CHAPTER 7

EFFECTS OF RS-ALBUTEROL ON THE DEVELOPMENT OF ANTIGEN-MEDIATED AIRWAY HYPERREACTIVITY IN GUINEA-PIGS

A version of this chapter has been accepted for publication in Clinical Reviews in Allergy and Immunology. Jafarian A., Handley D.A. and Biggs D.F.

7.1. INTRODUCTION

β_2-adrenoceptor agonists are widely used to treat bronchospasm in patients with asthma and chronic obstructive pulmonary disease. Although most of their pharmacologic activity resides in the R enantiomer (Brittain et al. 1973;Sanjar et al., 1990; Morley et al., 1991; Hoshiko et al., 1993; Mazoni et al., 1995), commercially available β_2-agonists are marketed as racemates. Use of β_2-agonists has been associated with increased morbidity and mortality in asthmatics (Barrett & Storm, 1995). In sensitized guinea-pigs, administration of RS-albuterol (1.0 mg kg^{-1} d^{-1}) for 6 days via an osmotic minipump induced increased airways' responsiveness to several bronchospastic agonists including histamine and leukotriene-C_4 (LTC_4) (Hoshiko & Morley, 1993). Also, in control and sensitized guinea-pigs, chronic administration of rac-fenoterol significantly increased airway responses to ACh both *in vivo* and in vitro (Wang et al., 1994). The purpose of this study was to determine whether sustained administration of RS-albuterol via an implanted osmotic minipump enhanced the increased airways' responsiveness to histamine and LTC_4 seen in guinea-pigs that had been passively immunized with α-OA Ab and challenged by inhalation of OA (2%) containing aerosols.

7.2. METHODS AND MATERIALS

7.2.1. Methods

7.2.1.1. Preparation of α-OA Ab containing serum

To obtain serum containing α-OA IgG Ab, guinea-pigs were immunized with a single ip injection of 20 mg kg^{-1} OA in saline. Twenty-one days later, on 8 consecutive days, animals were given pyrilamine (0.5 mg kg^{-1}, ip) and 30 min later inhaled OA (2%, in saline) aerosol (VIX Acorn™ nebulizer, compressed air at 15 psi) for up to 8 min. Guinea-pigs were carefully observed for the appearance of the signs of anaphylaxis. If these signs appeared, they were immediately removed from the exposure chamber and observed. If the signs persisted, they were placed in a chamber containing 100% oxygen. If the signs still persisted, epinephrine (0.5 mg kg^{-1}) was given subcutaneously (sc). Guinea-pigs developed a predominantly α-OA IgG-mediated reaction as defined by passive cutaneous anaphylaxis of their serum at various times post-injection and ELISA of their serum using anti-guinea-pig IgG, IgG$_1$ and IgG$_2$ antibodies (Campbell, 1992). On the 30th day after immunization, the guinea-pigs were anesthetized with pentobarbital (40-50 mg kg^{-1}) and exsanguinated under aseptic conditions via cardiac puncture (20 mL syringe, 16 G needle). The blood was placed in a sterile glass centrifuge tubes, sealed and centrifuged at 1,500 rpm for 10 min before being stored at 4°C overnight. Then, the tubes were centrifuged again (1,500 rpm, 10 min), the serum aspirated, pooled, placed in sterile polypropylene tubes and stored at -20°C. An aliquot of serum was used for determination of the titre of α-OA Ab. Serum was treated with ammonium sulfate to precipitate immunoglobulins, which were collected by centrifugation, dissolved in phosphate buffer (pH = 7.4) and dialyzed for 48 h against four changes of phosphate buffer. An aliquot of the dialyzed immunoglobulins was diluted and the titre of a-OA Ab determined. The titre of the α-OA Ab containing immunoglobulin solution was adjusted to a standard titre of 2,500 by addition of phosphate buffer. This standardized material was used for passive immunization (dose = 0.5 mL kg^{-1}).

7.2.1.2. Passive immunization of guinea-pigs

All guinea-pigs received two injections of standardized α-OA Ab solution prepared as described above (0.5 mL kg^{-1}, ip) 1 d apart as set out in the protocol below.

7.2.1.3. Aerosol exposure

Each group of guinea-pigs was exposed to OA (2%)- or saline-aerosol for 8 min 1 d after receiving each injection of α-OA Ab solution. Each animal was pretreated with pyrilamine (0.5 mg kg^{-1}, ip) 30 min before inhaling either aerosol.

7.2.1.4. Measurement of airways' responsiveness

Guinea-pigs were anesthetized with pentobarbital sodium (35-50 mg kg^{-1}, ip). Their tracheas were cannulated and they were connected to a rodent ventilator (Ugo Basile) that provided a tidal volume of 10 mL kg^{-1} at 20 breaths min^{-1}. Air flow was measured via a pneumotach (Fleisch 0000, Validyne MP45 differential pressure transducer) and intratracheal pressure relative to atmosphere via a pressure transducer (Validyne MP45). Signals were digitized and processed to yield breath-by-breath values of pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L). A catheter was placed in a jugular vein to give drugs iv. All animals received succinylcholine (0.1 mg kg^{-1}, iv) to prevent spontaneous breathing movements that would interfere with measurements. Increasing doses of histamine or LTC_4 were injected (iv) in increasing order of dose with recovery to baseline between injection of doses until R_L increased by at least 150% over baseline. Histamine was always given first.

7.2.1.5. Preparation of osmotic pumps

Sustained infusion of RS-albuterol was achieved by employing a sterile solution in saline deployed in ALZET™ osmotic pumps (2ML1, 10 mL h^{-1}, 7 d). Sterile saline alone containing citric acid was used as control vehicle. Individual pumps were prepared for each guinea-pig. Dose of RS-albuterol was 1.0 mg kg^{-1} d^{-1}. Solutions placed in the pumps were sterilized by filtration (0.22 mm syringe-end filter). Two mL of solution was placed into each pump. Pumps were primed by placing them in sterile saline at 24-26°C for 4-6 h before implantation. All procedures involved in preparation of the pumps were performed using aseptic technique.

The 2ML1 pumps' specifications state that they deliver 10 μL h^{-1}. Thus, for a guinea-pig weighing 1.0 kg, the pump must contain a solution of 4.16667 mg mL^{-1} RS-albuterol base. This solution needs to be diluted *pro rata* for guinea-pigs that weigh < 1.0 kg. A similar procedure was used for the control solution.

7.2.1.6. Implantation of pumps

The ALZET™ osmotic pumps containing the drug or control solution were implanted sc in the nuchal region under isoflurane anesthesia using aseptic surgical technique. The procedure mimicked that shown in the pump manufacturer's instructional videotape. The incision was closed with standard wound clips. All animals received buprenorphine (0.1 mg kg^{-1}, sc) immediately after pump implantation to control any pain after surgery.

7.2.1.7. Removal of pumps

The pump implanted in each animal was removed as soon as each guinea-pig was anesthetized for pulmonary measurements. The wound clips were removed, the incision opened and the pump removed with forceps.

7.2.1.8. Evaluation of pump function

The patency of the delivery portal emitting the drug solution was confirmed visually under a dissecting microscope (magnification = x 15). Then the flow moderator was removed and the fluid remaining in each pump was aspirated via a syringe and a blunt-tipped filling tube; the volume remaining in the pump was noted.

7.2.1.9. Bronchial alveolar lavage

After measurement of airway responsiveness, the lungs were lavaged with 2 x 5 mL PBS containing BSA (1%), at 24-26°C. The two lavage fluids were combined. Two Cytospin™ preparations were made and stained for differential cell counts.

7.2.1.10. Protocol

The experimental design is summarized in Table 7.1 and Fig. 7.1.

7.2.2. Animals

All procedures involving animals were examined and approved by the Health Sciences Laboratory Animal Welfare Committee, University of Alberta.

Twenty four female, Hartley-strain guinea-pigs, weight range 300-400 g, were purchased from Charles River Inc., St. Constant, Québec. They were shipped to Edmonton via air freight in filter top boxes. Upon arrival, they were randomly divided into four groups of six animals each and placed in cages in a laminar flow enclosure that supplied HEPA-filtered air. They were maintained on grids over trays of rock salt, fed standard guinea-pig chow supplemented with apples and allowed water *ad lib.*

7.2.3. Materials

Drugs used were: RS-albuterol base (Sepracor Inc. Marlborough, MA); albumin, chicken egg (ovalbumin) Grade V, citric acid, leukotriene-C_4, pyrilamine maleate and succinylcholine chloride (Sigma, St Louis, MO); buprenorphine hydrochloride (Temgesic®, Reckitt & Colman Pharmaceuticals, Hull, UK); histamine dihydrochloride (Fluka A.G., Buchs, Switzerland); isoflurane (Forane®, Ohmeda, Mississauga, ON); sodium pentobarbital (Euthanyl™, M.T.C. Pharmaceuticals, Markham, ON).

TABLE 7.1

Experimental design

GROUP	n	IMMUNIZATION	AEROSOL	RS-albuterol
1	6	+	SALINE	-
2	6	+	OVALBUMIN	-
3	6	+	SALINE	+
4	6	+	OVALBUMIN	+

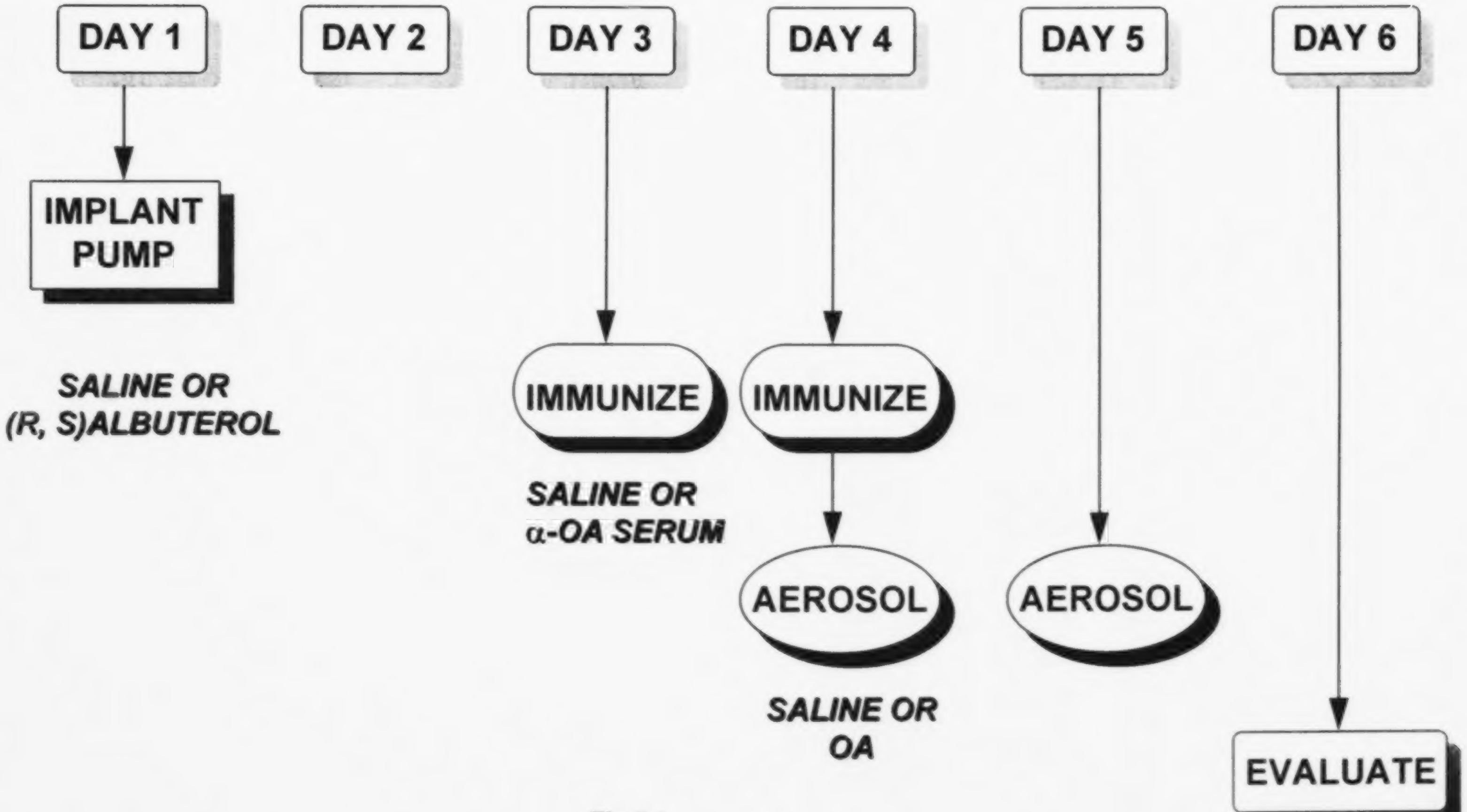

DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
IMPLANT PUMP
SALINE OR (R, S)ALBUTEROL
IMMUNIZE
IMMUNIZE
SALINE OR α-OA SERUM
AEROSOL
AEROSOL
SALINE OR OA
EVALUATE
HISTAMINE LTC_4

Fig 7.1
Schematic diagram illustrating the protocol. Animals in Group 1 were implanted with pumps on day 6,Group 2 on day 7, Group 3 on day 8 and Group 4 on day 9.

7.2.4. Statistical analyses

Differences among groups were analysed using Student's *t* test, one- and two way ANOVA, Kruskul-Wallis one-way ANOVA on ranks and Student-Newman-Keuls Test. SigmatStat™ was used to apply these tests. Significance was assumed at the 5% level.

7.3. RESULTS

7.3.1. Implantation of pumps

All guinea-pigs tolerated the procedure to implant the osmotic pumps well. At experiment, one animal from Group 2 showed some signs of infection at the implantation site. All other animals showed no signs of infection and the incision sites had all healed well during the time frame of the experiments.

7.3.2. Evaluation of pump function

Inspection of the emitting orifices of the pumps showed all to be patent and apparently functioning properly. Aspiration of the remaining solution in each pump revealed that less solution had been delivered to each animal than anticipated. Each pump should have delivered about 1.4 mL of solution (10 μL x 24 h x 6 d), but only about 1.2 mL, approximately 85% of the amount predicted, was delivered. Data for the pumps from the four Groups are shown in Table 2. There was no statistically significant differences among the pump volumes delivered among the four experimental groups.

TABLE 7.2

Volumes (mL) delivered (2.00 mL - volume aspirated) by each osmotic pump

ANIMAL #	GROUP 1	GROUP 2	GROUP 3	GROUP 4
1	1.10	1.10	1.10	1.10
2	1.10	1.30	1.20	1.30
3	1.00	1.00	1.25	1.20
4	1.10	1.20	1.20	1.30
5	1.24	1.15	1.25	1.20
6	1.40	1.20	1.20	1.30
MEAN ± SE	1.16 ± 0.13	1.16 ± 0.10	1.22 ± 0.02	1.26 ± 0.04
PERCENT PREDICTED	83	83	87	90

7.3.3. Effects noted upon aerosol exposure

All guinea-pigs were pretreated with pyrilamine (0.5 mg kg^{-1}, ip) 30 min before exposure to saline or OA aerosols. Groups 2 and 4 inhaled OA aerosols. None of the guinea-pigs in these Groups showed any signs of anaphylaxis and all animals were exposed to aerosols for 8 min on each of the two specified days.

7.3.4. Baseline measurements of R_L and E_L

There were no statistically significant differences among the baseline measurements of R_L and E_L. Data are summarized in Figs. 7.2A and 7.2B.

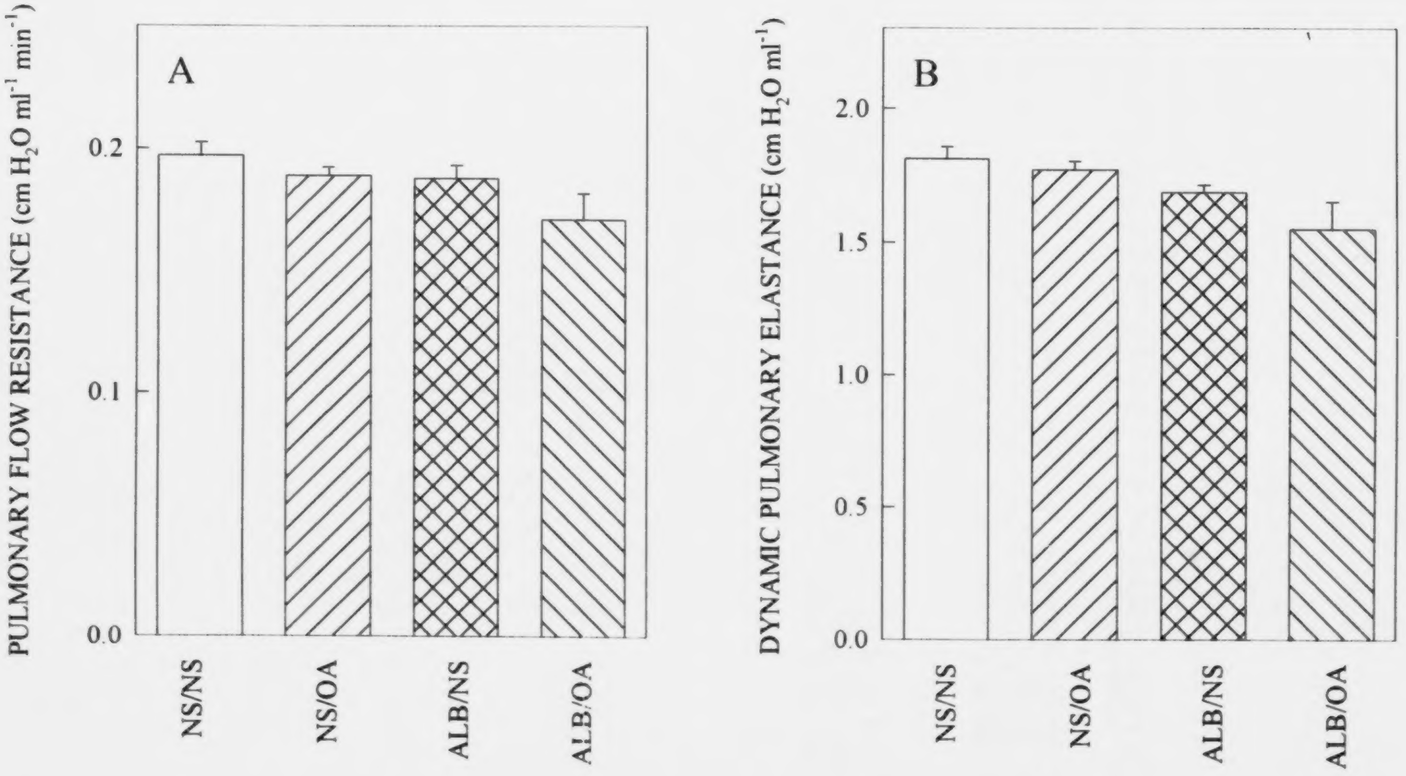


Fig. 7.2

Baseline values of pulmonary flow resistance (A) and dynamic pulmonary elastance (B) in Groups 1-4. There were no statistically significant differences in the values among the Groups.

7.3.5. Dose-response curves to histamine and LTC_4

Changes in R_L and E_L in response to graded doses of histamine and LTC_4 (both iv) in the four Groups are shown in Figs. 7.3-7.6. We first compared dose-response curves to histamine in guinea-pigs that had received α-OA serum (ip) and inhaled saline (Group 1) or inhaled OA (2%) aerosol (Group 2); in both groups the osmotic pumps contained control solution. For changes in R_L induced by histamine (iv), inhalation of OA aerosol induced about a two-fold, statistically significant ($p < 0.05$) shift of the dose-response curve to the left, in comparison with inhalation of saline aerosol. A similar shift was noted when LTC_4 was the agonist. However, the variance was such that the shift was not statistically significant. In our hands, LTC_4 had less than half the activity reported by previous workers (Hoshiko et al., 1993). Similar findings were noted with measurements of changes in E_L in response to histamine and LTC_4. Histamine was about twice as active in inducing changes in R_L than E_L; by contrast LTC_4 was equally active at inducing changes in R_L and E_L.

We next compared dose-response curves in guinea-pigs in Groups 1 and 3. Both groups received α-OA serum (ip); both groups inhaled saline. In Group 1 the osmotic pump contained control solution and in Group 3 it contained RS-albuterol. For the changes in R_L induced by histamine (iv), administration of RS-albuterol induced about a two-fold, statistically significant ($p < 0.05$) shift of the dose-response curve to the left, in comparison with control solution-treated controls. When LTC_4 was the agonist, significantly increased airways' responsiveness was noted only at the highest dose used ($1.8\ \mu g\ kg^{-1}$). Similar findings were noted when the effects of histamine and LTC_4 were compared on changes in E_L.

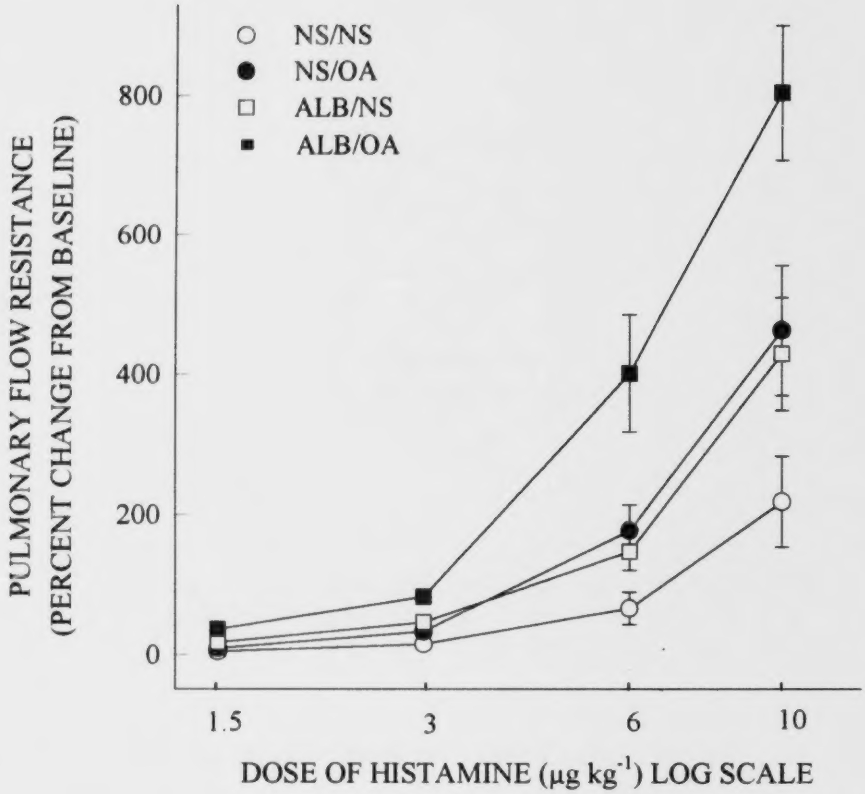


Fig. 7.3

Changes, as percent of baseline values, in pulmonary flow resistance in anesthetized, paralyzed guinea-pigs following injection (iv) of histamine in experimental Groups 1-4. Each point represents the mean ± SEM of 6 experiments. (* $p < 0.05$ - Group 1 vs 2; + $p < 0.05$ - Group 1 vs 3; # $p < 0.05$ - Group 2 vs 3; ** $p < 0.05$ - Group 3 vs 4)

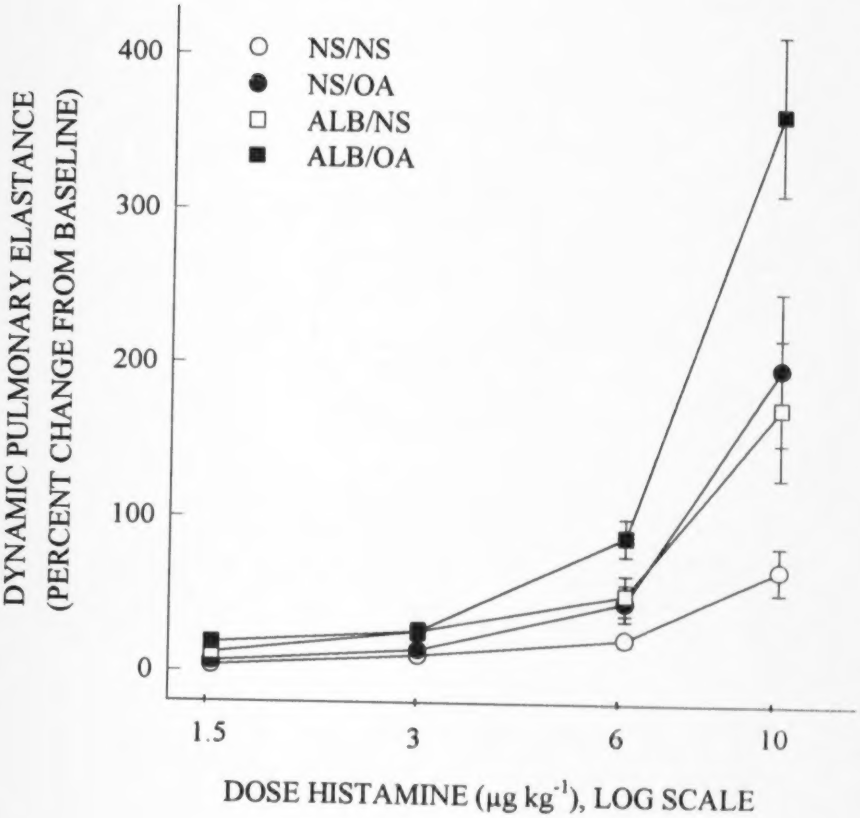


Fig. 7.4

Changes, as percent of baseline values, in dynamic pulmonary elastance in anesthetized, paralyzed guinea-pigs following injection (iv) of histamine in experimental Groups 1-4. Each point represents the mean ± SEM of 6 experiments. (* $p < 0.05$ - Group 1 vs 2; + $p < 0.05$ - Group 1 vs 3; # $p < 0.05$ - Group 2 vs 3; ** $p < 0.05$ - Group 3 vs 4)

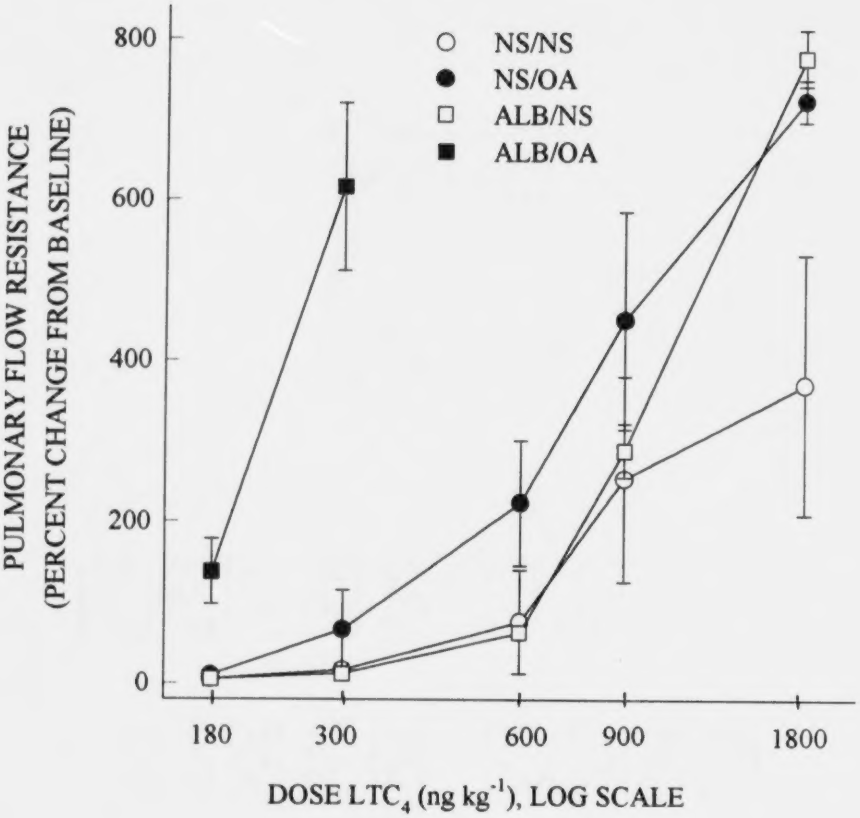


Fig. 7.5

Changes, as percent of baseline values, in pulmonary flow resistance in anesthetized, paralyzed guinea-pigs following injection (iv) of LTC_4 in experimental Groups 1-4. Each point represents the mean ± SEM of 6 experiments. (* $p < 0.05$ - Group 1 vs 2; + $p < 0.05$ - Group 1 vs 3; # $p < 0.05$ - Group 2 vs 3; ** $p < 0.05$ - Group 3 vs 4)

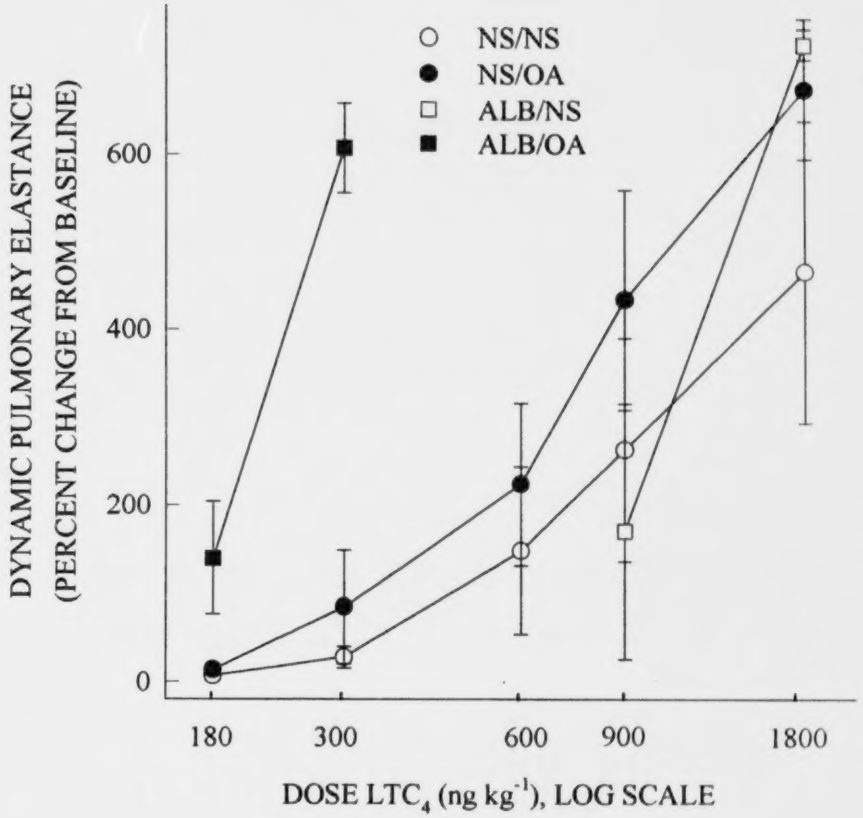


Fig. 7.6

Changes, as percent of baseline values, in dynamic pulmonary elastance in anesthetized, paralyzed guinea-pigs following injection (iv) of LTC_4 in experimental Groups 1-4. Each point represents the mean ± SEM of 6 experiments. (* $p < 0.05$ - Group 1 vs 2; + $p < 0.05$ - Group 1 vs 3; # $p < 0.05$ - Group 2 vs 3; ** $p < 0.05$ - Group 3 vs 4)

Lastly, we compared dose-response curves in guinea-pigs in Groups 2 and 4. Both groups received α-OA serum (ip); both groups inhaled OA aerosols. In Group 2, the pump contained control solution, for Group 4, it contained RS-albuterol. For changes in R_L induced by histamine, administration of RS-albuterol and inhalation of OA aerosol induced about a two-fold, statistically significant ($p < 0.05$), shift of the dose-response curve to the left, compared to animals that inhaled OA aerosol but received no RS-albuterol. Two-way ANOVA indicated that the effects of OA aerosol and RS-albuterol on airways' responsiveness to histamine were additive. Similar findings were noted when the effects of histamine on E_L were examined. When LTC_4 was the agonist, about a four-fold, statistically significant ($p < 0.05$) shift of the dose-response curves was noted. By contrast, guinea-pigs that had received control solution and inhaled OA aerosol (see Figs. 7.3-7.6, Groups 1 and 2) showed about a two-fold, and animals that had received (rac)-albuterol (see Figs. 7.3-7.6, Groups 1 and 3) a minimal shift of the dose-response curve. Similar findings were noted when the effects of histamine on E_L were examined. Two-way ANOVA indicated that the effects of OA aerosol and RS-albuterol on airways' responsiveness to LTC_4 were synergistic.

7.3.6. Differential cell counts

Data are summarized in Figs. 7.7A, B and C. Analyses of the findings were complicated by the fact that occasional animals showed profound eosinophilia in their lavage fluid. Nevertheless, the data suggest that RS-albuterol may reduce the number of eosinophils found in these animals.

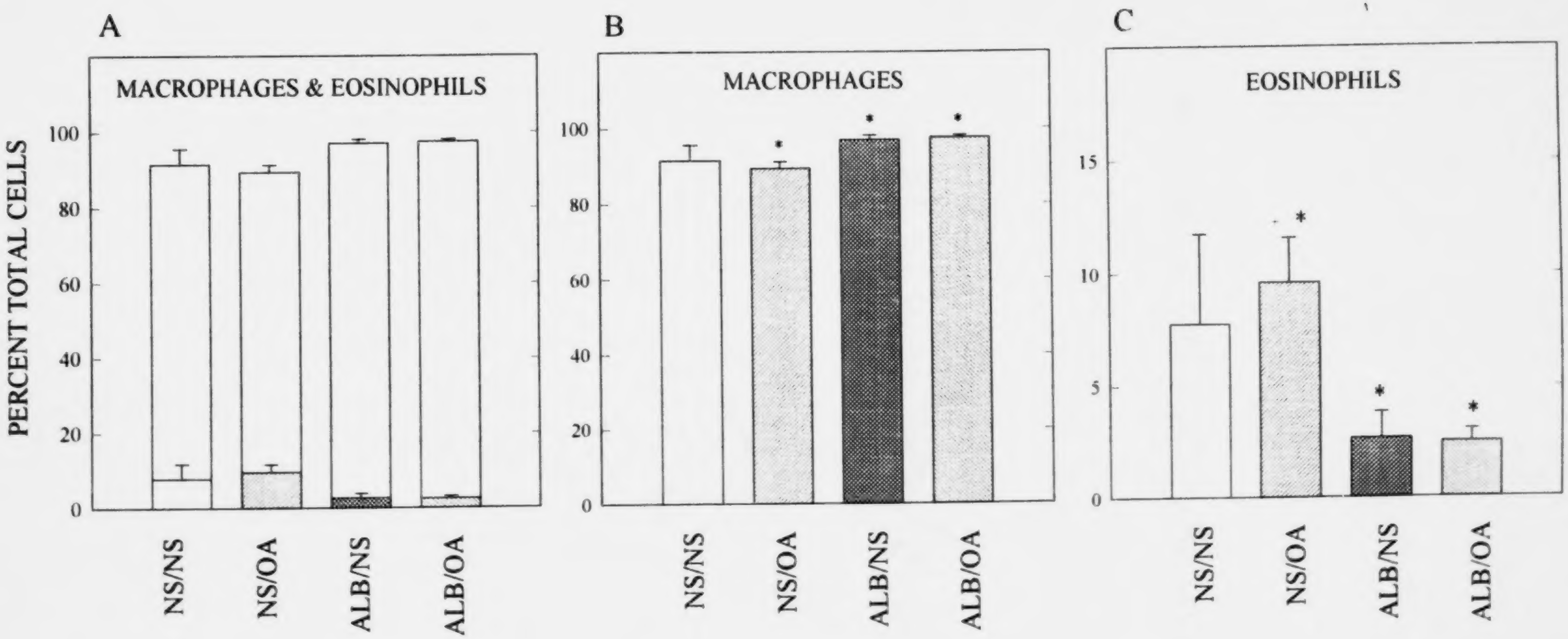


Fig. 7.7

Summary of data obtained from differential cell counts in the four groups. A: Macrophage and eosinophil counts as percent cells superimposed upon one another bigger columns are macrophages. B: Macrophages as percent total cells; C: Eosinophils as percent total cells. Each bar shows the mean ± SEM of counts from 6 guinea-pigs. (* $p < 0.05$)

7.4. DISCUSSION

These findings confirm our earlier reports (Ladenius, 1993) that passive immunization of guinea-pigs to OA and subsequent challenge with OA aerosol(s) significantly increases airways' responsiveness to histamine (iv), compared to a similarly treated control group that had inhaled saline aerosols. Interestingly, in the experiments reported here, generally similar findings were noted with LTC_4. These data confirm others' observations (Hoshiko et al., 1993; Hoshiko & Morley, 1993) but the dose-response curves we obtained were markedly steeper than those authors reported and we found LTC_4 to have about one half to one third the bronchospastic activity reported (Hoshiko et al., 1993; Hoshiko & Morley, 1993).

Our findings show clearly that RS-albuterol infused via an osmotic pump for 6 days increased airways' responsiveness to histamine and to LTC_4, in comparison with the appropriate control groups. Interestingly, the effects of OA inhalation and RS-albuterol appeared to be additive when histamine was the agonist and synergistic when LTC_4 was the agonist. At no time did we observe that infusion of RS-albuterol reduced OA aerosol-induced airway hyperresponsiveness. It clearly enhanced it with either agonist and to an equal or much greater extent than that seen following immunization and antigen challenge. Also, comparison of baseline measurements of R_L and E_L showed that infusion of RS-albuterol for 6 days did not signficantly alter these values. Thus, despite its well established airway smooth muscle relaxant effects, RS-albuterol did not induce either a decrease or an increase in airway tone as assessed by these measurements.

The findings from the differential cells counts of lavage fluid suggest that infusion of RS-albuterol may reduce eosinophil counts. However, the high eosinophil counts found in some animals in Groups 1 and 2 suggest that eosinophilia may be endemic in these animals' lungs. "Spontaneous" pulmonary eosinophilia has been a seasonal problem with guinea-pigs (unpublished observations). Further experiments are required to validate these interesting observations with RS-albuterol.

The mechanisms that underlie RS-albuterol's ability to enhance airways' responsiveness were not defined in these experiments. In humans, stereoselective metabolism of RS-albuterol that results in accumulation of the distomer S-albuterol has been reported (Tan & Soldin, 1987; Walle et al., 1993) and it is possible that this occurs in guinea-pigs as well. Others (Sanjar et al., 1990; Mazzoni et al., 1995) have associated an increased mortality from anaphylaxis in immunized guinea-pigs after inhalation of antigen with pretreatment of the animals with β_2-agonists. This was attributed to the increased toxicity from selective accumulation of the distomers. Interestingly, these workers reported that in their guinea-pigs any increased airways' responsiveness to agonists could be prevented by bilateral vagotomy or ketotifen (Hoshiko et al., 1993; Mazzoni et al., 1995).

In conclusion, these experiments demonstrate clearly that RS-albuterol markedly enhanced airways' responsiveness to histamine in passively immunized guinea-pigs that inhaled saline or OA containing aerosols. By contrast, RS-albuterol significantly enhanced airways' responsiveness to LTC_4 only in passively immunized guinea-pigs that had inhaled OA. Two-way ANOVA showed that the effects of OA aerosol inhalation and RS-albuterol on airways' responsiveness were additive when histamine was the agonist and synergistic when LTC_4 was the agonist. These findings suggest that RS-albuterol may have more adverse effects in asthmatics than in normals.

CONCLUSIONS AND SUMMARY

To characterize the involvement of sensory C-fibres in inducing bronchoconstriction in guinea-pigs, we first established reliable methods for assessing airways' responsiveness. Thus, the natural variation in responsiveness to vagal stimulation and to bronchospastic agonists in guinea-pigs was examined. The stimulus parameters used appeared to selectively activate preganglionic parasympathetic nerves as atropine and hexamethonium eliminated the responses.

Measurements of R_L and E_L suggested that vagal stimulation and the various agonists selectively altered these measures of lung responsiveness. Thus, vagal stimulation induced stimuli-dependent changes in R_L. However, methacholine, histamine, 5HT, SP and NKA induced dose-dependent changes in R_L and E_L. These differences suggest that these agonists affect both the conducting airways and the distensible airways, whereas vagal stimulation mainly affect the conducting airways. Based on our findings, vagal stimulation and/or methacholine, histamine, 5HT, SP and NKA (iv) appear to be appropriate for comparing airways' responsiveness as responsiveness to these modalities was reproducible among animals.

The next step was to produce large quantities of α-SP MAb and to characterize this Ab. Two procedures were employed for production of α-SP MAb, the classical bulk cell culture method and the hollow-fibre bioreactor. SDS-PAGE revealed that α-SP MAb obtained from bioreactor was about 80% pure, so that it was used for *in vitro* and *in vivo* experiments without any purification. By contrast, α-SP MAb obtained by the classical bulk culture method needed to be purified. Neither protein A- nor protein G-sepharose gave a satisfactory absorption and separation of the α-SP MAb even after preliminary separation of protein with ammonium sulphate. However, thiophilic gel columns provided excellent absorption of α-SP MAb from raw supernatant and provided a satisfactory purification method.

The relative affinities of the α-SP MAb for SP, NKA, CGRP and SP fragments were estimated by inhibition ELISA. The order of potency of these peptides to inhibit binding of α-SP MAb to BSA-SP conjugate was: $SP^{6-11} > SP^{7-11} > SP = SP^{2-11} = SP^{3-11} = SP^{4-11} = SP^{5-11} > SP^{8-11} > SP^{9-11}$. N-terminal SP fragments, mono- or di-peptides derived from SP, CGRP and selective NKR antagonists showed minimal affinity for α-SP MAb. These experiments show that the monoclonal α-SP MAb derived from the rat/mouse heterohybridoma NC1/34 is directed against epitopic sites on SP's C-terminal. These findings suggest that amino acids 6 and 7, and, to a lesser extent, 8 and 9, are important in determining affinity. Solid phase peptide syntheses on membranes were used to try and confirm the conclusions drawn from measurements of relative affinities by inhibition ELISA. Dot immunoassay of all these peptides revealed that none bound the monoclonal α-SP MAb, but all of the peptides bound the polyclonal α-SP serum used as a positive control. These findings indicate the importance of an accessible carboxy group at the C-terminal of the peptide for binding to the monoclonal α-SP MAb.

In our experiments, the α-SP MAb bound neither the NK1- nor the NK2-receptor antagonist suggesting that the paratopic site on the antibody bears little resemblance to the binding sites on NKR.

α-SP MAb given iv to guinea-pigs specifically prevented increases in R_L and E_L induced by SP or NKA (iv) but not by capsaicin. However, when α-SP MAb was given ip and iv, it decreased capsaicin-induced changes in R_L and E_L. The findings with this specific monoclonal α-SP MAb indicate that passive immunization of guinea-pigs, with α-SP MAb prevents the bronchospastic effects of SP and NKA, *in vivo*, and confirm that the endogenous release of the neurokinins, SP and NKA, mediates bronchoconstriction induced by capsaicin.

In the next step, we characterized neurokinin-mediated responses in guinea-pigs by comparing the actions of selective NK1 and NK2 receptor antagonists against SP-, SP fragments-, selective NKR agonists-, NKA- and capsaicin-induced bronchoconstriction. The order of potency of SP and its fragments in inducing bronchospasm was: $SP^{4-11} >> SP^{2-11} = SP^{3-11} = SP^{5-11} > SP = SP^{6-11}$. N-terminal fragments of SP were devoid of bronchospastic activity. Our findings indicated that bronchospastic activity of SP depends on its C-terminal and that the minimal requirement for inducing bronchospastic activity is 6 amino acids in the C-terminal of SP.

CP 96,345 or CP 99,994 blocked responses to a selective NK1 receptor agonist and greatly reduced responses to SP and SP fragments. SR 48,968 blocked responses to NKA, a selective NK2 receptor agonist and significantly reduced responses to SP^{4-11}. These findings suggest that SP, SP^{5-11}, SP^{3-11}, SP^{2-11} and SP^{6-11} induce bronchoconstriction mainly via NK1 receptors; SP^{4-11} acts via NK1 and NK2 receptors. A combination of NK1 and NK2 receptor antagonists caused greater rightward shift of the dose-response curves to SP, SP fragments and NKA suggesting that bronchoconstriction induced by SP, SP fragments or NKA is mediated via both NK1 and NK2 receptors in guinea-pig.

To further study the mechanisms underlying the effects of activation of sensory C-fibres, we looked at endogenously released TK, SP and NKA, via capsaicin. In the experiments reported here, neither atropine, nor bilateral vagotomy, nor mecamylamine reduced bronchospastic responses to capsaicin. Thus, we have no evidence implicating central or axonal reflex-mediated parasympathetic effects in capsaicin-induced bronchospasm. Pretreatment of animals with the ganglionic blocker mecamylamine or the non-specific β-receptor blocker nadolol enhanced responses to capsaicin and shifted dose-response curves significantly to the left. The increase in baseline after giving nadolol could account, in part, for the enhancement of bronchopulmonary responses to capsaicin. Paradoxically, combinations of nadolol and mecamylamine showed an additive enhancement of capsaicin-induced bronchospasm. Thus, in our experimental situation, the airways have sympathetic tone. "Nonspecific" effects of nadolol could account for additive effect of combinations of nadolol and mecamylamine and the increase in baseline values of R_L and E_L.

The selective NK1 receptor antagonist CP 96,345 had no effect on bronchopulmonary responses to capsaicin. However, the selective NK2 receptor antagonist SR 48,968 greatly reduced bronchopulmonary responses to capsaicin but did not eliminate them. A combination of NK1 and NK2 receptor antagonists was necessary to eliminate them. Activation of NK2 receptors dominates capsaicin's pulmonary effects. The synergistic effects of combination of selective NK1 and NK2 receptor antagonist leads to the assumption that there may be conformational compatibility between each antagonist and neurokinin receptors. Thus, binding to one receptor may lead to specific changes which are different from changes which occure when both receptors are occupied or binding of one antagonist to its receptor may facilitate the binding of the other antagonits to its receptor. Also it is possible that both NK1 and NK2 receptors are located on the same cell or NK1 and NK2 receptor antagonists act on different sites of the same protein.

Our findings revealed that hCGRP in doses that significantly decreased mean arterial blood pressure, had no effect on changes in R_L and E_L induced by SP, NKA or capsaicin. The hCGRP antagonist, in a dose that reduced the depressor effect of hCGRP, *increased* bronchopulmonary responses to capsaicin. The ability of $hCGRP^{8-37}$ to increase capsaicin-induced bronchoconstriction may be due to its blocking action on the effects of CGRP released from capsaicin sensitive C-fibre afferents. Although we were unable to demonstrate that hCGRP blocked bronchoconstriction induced by exogenous agonists, these findings suggest that the capsaicin-induced release of CGRP may modulate bronchospasm induced by the endogenously released SP and NKA.

Finally, we studied an animal model of asthma by passively immunizing guinea-pigs with an α-OA Ab. We sought to determine whether sustained administration of RS-albuterol enhanced the increased airways' responsiveness to histamine and LTC_4 in guinea-pigs. These findings showed that passive immunization of guinea-pigs to OA and subsequent challenge with OA aerosol(s) significantly increases airways' responsiveness to histamine and LTC_4. RS-albuterol increased airways' responsiveness to histamine and to LTC_4, in comparison with the appropriate control groups. The effects of inhalation and RS-albuterol appeared to be additive when histamine was the agonist and synergistic when LTC_4 was the agonist. Despite its well established airway smooth muscle relaxant effects, RS-albuterol did not induce either a decrease or an increase in airway tone as assessed by these measurements. The findings from the differential cells counts of lavage fluid suggest that infusion of RS-albuterol may reduce eosinophil counts. However, the high eosinophil counts found in some animals in control groups suggest that eosinophilia may be endemic in these animals' lungs. These findings suggest that RS-albuterol may have more adverse effects in asthmatics than in normals.

REFERENCES

Abbas, A.K., Lichtman, A.H. and Pober, J.S. 1994. Antibodies and antigens . *In: Cellular and Molecular Immunology.* pp: 34-64. W.B. Saunders Company, Pensylvania.

Abdel-Latiff, A.A. 1986. Calcium mobilizing receptors: phosphoinositides and the generation of second messengers. Pharmacol. Rev. **38**: 227-71.

Adnot, S., Cigarini, I., Herigault, R. and Harf, A. 1991. Effects of substance P and calcitonin gene-related peptide on the pulmonary circulation. J. Appl. Physiol. **70**: 1707-1712.

Adocock, I.M., Peters, M., Gelder, C., Shirasaki, H., Brown, C.R. and Barnes, P.J. 1993. Increased tachykinin receptor gene expression in asthmatic lungs and its modulation by steroids. J. Molecul. Endocrin. **11**: 1-7.

Advenier, C., Cerrina, J., Duroux, P., Floch, A., Paradel, J. and Renier, A. 1983. Sodium chromoglycate, verapamil and nicardipine antagonism to leukotriene D_4 bronchoconstriction. Br. J. Pharmacol. **78**: 301-306.

Advenier, C., Rouissi, N. and Neliat, G. 1992. Neurokinin A (NK2) receptor revisited with SR 48,968, a potent non-peptide antagonist. Biochem. Biophys. Res. Commun. **184**: 1418-1424.

Al-Kazawini, S.J., Craig, R.K. and Marshall, I. 1986. Postjunctional inhibition of contractor responses in the mouse vas deferens by rat and human calcitonin gene-related peptide. Br. J. Pharmacol. **88**: 173-180.

Alving, K., Fornhem, C. and Lundberg, J.M. 1993. Pulmonary effects of endogenous and exogenous nitric oxide in the pig: relation to cigarette smoke inhalation. Br. J. Pharmacol. **110**: 739-746.

Alving, K., Fornhem, C., Weitzberg, E. and Lundberg, J.M. 1992. Nitric oxide mediates cigarette smoke-induced vasodilatory responses in the lung. Acta Physiologica Scandinavica. **146**: 407-8.

Alexander, H.L. and Paddock, R. 1921. Bronchial asthma: response to pilocarpine and epinephrine. Arch. Int. Med. **27**: 184-191.

Amann, R., Donnerer, J. and Lembeck, F. 1990. Activation of primary afferent neurons by thermal stimulation - influence of ruthenium red. Naunyn Scheimdeberg's Arch. Pharmacol. **341**: 108-113.

American Thoracic Society. 1987. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am. Rev. Respir. Dis. **136**: 225-244.

Andersen, B.G. and Gruenberg, M.L. 1987. Optimization techniques for the production of monoclonal antibodies utilizing hollow-fibre technology. *In: Commercial Production of Monoclonal Antibodies, a Guide for Scale Up* (Seaver, S.S., ed.) pp: 175-179, Marcel Dekker Inc., New York and Basel.

Andersson, R.G.G. and Grundstrom, N. 1983. The excitatory non-cholinergic, non-adrenergic nervous system of the guinea-pig airways. Eur. J. Respir. Dis. **64**: 141-157.

Arita, H., Sakamoto, M., Hirokawa, Y. and Okado, N. 1993. Serotonin innervation patterns differ among the various medullary motoneuronal groups involved in upper airway control. Exp. Brain Res. **95**: 100-110.

Atherton, E. and Sheppard, R.C. 1989. Solid Phase Peptide Synthesis - a Practical Approach. IRL Press at Oxford University Press ISBN 019-963066-6.

Bai, T.R., Bramley, A.M., Schellenberg, R.R. and Pare, P.D. 1992. Nitric oxide mediates non-adrenergic, non-cholinergic inhibitory neural response in human bronchial smooth muscle. Am. Rev. Respir. Dis. **145**: A 383.

Ballati, L., Evangelista, S., Maggi, C.A. and Manzini, S. 1992. Effects of selective tachykinin receptor antagonists on capsaicin- and tachykinin-induced bronchospasm in anesthetized guinea-pigs. Eur. J. Pharmacol. **214**: 215-221.

Barnes, P.J. 1984. β-Adrenoceptors in lung tissue. *In: β-Adrenoceptors in Asthma*. pp. 67-92, (Morley, J. ed.) Academic Press, London.

Barnes, P.J. 1986a. Asthma as an axon reflex. Lancet. **I**: 242-245.

Barnes, P.J. 1986b. State of art. Neural control of human airways in health and disease. Am. Rev. Respir. Dis. **134**: 1289-1314.

Barnes, P.J. 1986c. Endogenous catecholamines and asthma. J. Clin. Immunol. **77**: 791-795.

Barnes, P.J. 1986d. Non-adrenergic, non-cholinergic control of human airways. Arch. Int. Pharmacodyn. Ther. **280 Suppl**: 208-228.

Barnes, P.J. 1987. Adrenergic regulation of airway function. *In : The Airways: Neural Control in Asthma and Airway Disease* (Barnes, P.J. ed.). pp: 57-85, Marcel Dekker, New York.

Barnes, P.J. 1988. Neuropeptides and airway smooth muscle. Pharmacol. Ther. **36**: 119-129.

Barnes, P.J. 1991a. Neuropeptides and asthma. Am. Rev. Respir. Dis. **143**: S28-S32.

Barnes, P.J. 1991b. Neural control of airway smooth muscle. *In: The Lung Scientific Foundation (Crystal, R.G. and West, J.B., eds.)* pp: 903-916, Raven Press, New York.

Barnes, P.J. 1992. Neural mechanisms in asthma. Br. Med. Bull. **48**: 149-168.

Barnes, P.J. 1993. Muscarinic receptor subtypes in airways. Life Sci. **52**: 521-527.

Barnes, P.J., Basbaum, C.B. and Nadel, J.A. 1983. Autoradiographic localization of autonomic receptors in airway smooth muscle. Am. Rev. Respir. Dis. **127**: 758-762.

Barnes, P.J. and Dixon, C.M.S. 1984. The effect of inhaled vasoactive intestinal peptide on bronchial hyperreactivity to histamine in humans. Am. Rev. Respir. Dis. **130**: 162-166.

Barnes, P.J., Cuss, F.M. and Palmer, J.B. 1985. The effect of airway epithelium on smooth muscle contractility in bovine trachea. Br. J. Pharmacol. **86**: 685-692.

Barnes, P.T., Ind, P.W. and Brown, M.J. 1982. Plasma histamine and catecholamines in stable asthmatic subjects. Clin. Sci. **62**: 661-665.

Barnes, P.J., Karliner, J., Hamilton, C. and Dollery, C. 1979. Demonstration of α-adrenoceptors in guinea-pig lung using 3H-prazosin. Life Sci. **25**: 1207-1214.

Barnes, P.J. and Liew, F.Y. 1995. Nitric oxide and asthmatic inflammation. Immunol. Today **16**: 128-130.

Barnes, P.J., Wilson, N.M. and Vickers, H. 1981. Prazosin, an α-adrenoceptor antagonist, partially inhibits exercise-induced asthma. J. Allergy Clin. Immunol. **68**: 411-419.

Barrett, T.E. and Storm, B.L. 1995. Inhaled beta-adrenergic receptor agonists in asthma: more harm than good? Am. J. Respir. Crit. Care Med. **151**: 574-577.

Bartho, L., Holzer, P., Lembec, F. and Szolcsanyi, J. 1982. Evidence that the contractile response of the guinea-pig ileum to capsaicin is due to release of substance P. J. Physiol. **332**: 157-167.

Bartho, L., Koczan, G., Holzer, P., Maggi, C.A. and Szolcsanyi, J. 1991. Antagonism of the effects of calcitonin gene-related peptide and capsaicin on the guinea-pig isolated ileum by human α-calcitonin gene-related peptide (8-37). Neurosci. Lett. **129**: 156-159.

Bartho, L., Koczan, G. and Maggi, C.A. 1993. Studies on the mechanism of the contractile action of rat calcitonin gene-related peptide and of capsaicin on the guinea-pig ileum: effect of hCGRP (8-37) and CGRP tachyphylaxis. Neuropeptides **25**: 325-329.

Baudouin, S.V., Altman, T.J. and Johnson, A.J. 1988. Prazosin in the treatment of chronic asthma. Thorax **43**: 385-387.

Belew, M., Juntti, N. and Porath, J. 1987. A one step purification method for monoclonal antibodies based on salt promoted adsorption chromatography on a thiophilic absorbent. J. Immunol. Meth. **102**: 173-182.

Belvisi, M.G., Patacchini, R, Barnes, P.J. and Maggi, C.A. 1994. Facilitatory effects of selective agonists for tachykinin receptors on cholinergic transmission: evidence for species differences. Br. J. Pharmacol. **111**: 103-110.

Benjamin, N., Dollery, C.T., Fuller, R.W., Larkin, S. and McEwan, J. 1987. The effects of calcitonin gene-related peptide and substance P on resistance and capillary vessels. Br. J. Pharmacol. **90 Suppl**: 43.

Berenstein, M.L., Berker, E. and Cullell, M. 1957. The bronchomotor effects of certain intravenous barbiturates on vagal stimulation in dogs. Anesthesiology **18**: 866-870.

Bertrand, C., Nadel, J.A., Graf, P.D. and Geppetti, P. 1993. Capsaicin increases airflow resistance in guinea-pigs in vivo by activating both NK2 and NK1 tachykinin receptors. Am. Rev. Respir. Dis. **148**: 909-914.

Bevan, S. and Yeast, J.C. 1989. A potent activated sustained inward current in rat dorsal ganglion neurons. Soc. Neurosci. **15**: 1152.

Biggs, D.F. and Goel, V. 1985. Does capsaicin cause reflex bronchospasm in guinea-pigs? Eur. J. Pharmacol. **115**: 71-80.

Black, J.W. and Shankley, N.P. 1995. Inverse agonists exposed. Nature **374**: 214-215.

Bloom, J.W., Yamamura, H.I., Baumgartener, C. and Halonen, M. 1987. A muscarinic receptor with high affinity for pirenzepine mediates vagally induced bronchoconstriction. Eur. J. Pharmacol. **133**: 21-27.

Borson, D.B., Corrales, R., Verson, S., Gold, M., Viro, N., Caughey, G., Ramachandran, J. and Nadel, J.A. 1987. Enkephalinase inhibitors potentiate substance P-induced secretion of 35SO4-macromolecules from ferret trachea. Exp. Lung Res. **12**: 21-36.

Boushey, H.A., Holtzman, M.J., Sheller, A.R. and Nadel, J.A. 1980. Bronchial hyperreactivity. Am. Rev. Respir. Dis. **121**: 389-409.

Boyle, S., Guard, S., Hodgson, J., Horwell, D.C., Howson, W., Hughes, J. and McNight, A. 1994. Rational design of high affinity tachykinin NK2 receptor antagonists. Bioorganic Med. Chem. **2**: 101-113.

Brain, S.D. and Williams, T.J. 1985. Inflammatory oedema induced by synergism between calcitonin gene-related peptide and mediators of increased vascular permeability. Br. J. Pharmacol. **85**: 855-859.

Brain, S.D., Williams, T.J., Tippins, J.R., Morris, H.R. and MacIntyre, I. 1985. CGRP is a potent vasodilator. Nature **313**: 54-56.

Briscoe, W.A. and DuBois, A.B. 1985. The relationship between airway resistance, airway conductance, and lung volume in subjects of different age and body size. J. Clin. Invest. **37**: 1279-1285.

British Thoracic Society. 1990. Guidelines for management of asthma in adults: I - Chronic persistent asthma. Br. Med. J. **301**: 651-653.

Brittain, R.T., Farmer, J.B. and Marshall, R.J. 1973. Some observations on the β-adrenoceptor agonist properties of isomers of salbutamol. Br. J. Pharmacol. **48**: 144-147.

Brody, M.J. and Kadowitz, P.J. 1974. Prostaglandins as modulators of the autonomic nervous system. Fed. Proc. **33**: 48-60.

Brown, R.H., Herold, C.J., Hirshman, C.A., Zerhouni, E.A. and Mitzner, W. 1991. In vivo measurements of airway reactivity using high-resolution computed tomography. Am. Rev. Respir. Dis. **144**: 208-212.

Brown, R., Ingram, R.H. and McFadden, E.R. 1978. Effects of prostaglandin $F_{2\alpha}$ on lung mechanics in nonasthmatic and asthmatic subjects. J. Appl. Physiol. **44**: 150-155.

Brugger, F., Evans, R.H. and Olpe, H. 1988. The effect of the excitatory amino acid antagonists and capsaicin on spinal reflexes of the rodent recorded in vitro. J. Physiol. **401**: 59P.

Brunelleschi, S., Ceni, E., Fantozzi, R. and Maggi, C.A. 1992. Evidence for tachykinin NK2B-like receptors in guinea-pig alveolar macrophages. Life Sci. 51: PL177-PL181.

Buck, S.H. and Burcher, E. 1986. Pharmacologic characterization and autoradiographic distribution of binding sites for iodinated tachykinins in the rat central nervous system. Peptides **7**: 1109-1120.

Buck, S.H., Burcher, E., Shults, C.W. and O'Donohue, T.L. 1984. Novel pharmacology of substance K-binding sites: a third type of tachykinin receptor. Science **226**: 987-989.

Burcher, E., Buck, S.H., Lovenberg, W. and O'Donohue, T.L. 1986. Characterization and autoradiographic localization of multiple tachykinin binding sites in gastrointestinal tract and bladder. J. Pharmacol. Exp. Ther. **236**: 819-831.

Cadieux, A. and Lanoue, C. 1990. Inhibition by calcitonin gene-related peptide of agonist-induced bronchoconstriction in various mammals including man. Agents Actions **31 Suppl**: 211-214.

Cadieux, A., Lanoue, C., Sirois, P. and Barabe, J. 1990. Carbamylcholine- and 5-hydroxyltryptamine-induced contraction in rat isolated airways: inhibition by calcitonin gene-related peptide. Br. J. Pharmacol. **101**: 193-199.

Cameron, A.R., Johnston, C.D., Kirkpatrick, C.T. and Kirkpatrick, M.C.A. 1983. The quest for the inhibitory neurotransmitter in bovine tracheal smooth muscle. J. Exp. Psychol. **68**: 413-426.

Campbell, J.W. 1992. Experimental asthma in guinea pigs. M.Sc. Thesis, University of Alberta.

Carstairs, J.R., Nimmo, A.J. and Barnes, P.J. 1985. Autoradiographic visualization of β-adrenoceptor subtypes in human lung. Am. Rev. Respir. Dis. **132**: 541-547.

Cascieri, M.A., Huang, R.R., Fong, T.M., Cheung, A.H., Sadowski, S., Ber, E. and Strader, C.D. 1992. Determination of the amino acid residues in substance P conferring selectivity and specificity for the rat neurokinin receptors. Molecular Pharmacol. **41**: 1096-1099.

Cascieri, M.A. and Liang, T. 1983. Characterization of the substance P receptor in rat brain cortex membranes and the inhibition of radioligand binding by guanine nucleotides. J. Biol. Chem. **258**: 5158-5164.

Caughey, G.H., Leidig, F., Viro, N.F. and Nadel, J.A. 1988. Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase. J. Pharmacol. Exp. Ther. **244**: 133-137.

Ceccatelli, S., Lundberg, J.M., Fahrenkrug, J., Bredt, D.S., Syndre, S.H. and Hokfelt, T. 1992. Evidence for involvement of nitric oxide in regulation of hypothalamic portal blood flow. Neurosci. **51**: 769-772.

Cerrina, J., Ladurie, M.L., Labat, C., Raffestin, B., Bayol, A. and Brink, C. 1986. Comparison of human bronchial muscle response to histamine in vivo with histamine and isoproterenol agonists in vitro. Am. Rev. Respir. Dis. **134**: 57-61.

Chahl, L.A. 1989. The effect of ruthenium red on the response of guinea-pig ileum to capsaicin. Eur. J. Pharmacol. **169**: 241-247.

Chausow, A.M. and Banner, A.S. 1983. Comparison of the tussive effects of histamine and methacholine in humans. J. Appl. Physiol. **55**: 541-546.

Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., Fukase, M., Noda, T. and Fujita, T. 1989. Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). Am. J. Physiol. 256: E331-E335.

Chipkin, R.E., Berger, J.G., Billard, W., Iorio, L., Chapman, R. and Barnett, A. 1988. Pharmacology of SCH 34826, an orally active enkephalinase inhibitor analgesic. J. Pharmacol. Exp. Ther. **245**: 829-838.

Chow, A.L. 1986. Analysis of airway responses to vagal stimulation in guinea-pig. M.Sc. Thesis, University of Alberta.

Chung, K.F., Evans, T.W., Graf, P.D. and Nadel, J.A. 1985. Modulation of cholinergic neurotransmission in canine airways by thromboxane mimetic U46619. Eur. J. Pharmacol. **117**: 373-375.

Clague, J.R., Sternini, C. and Brecha, N.C. 1985. Localization of calcitonin gene-related peptide-like immunoreactivity in neurons of rat gastrointestinal tract. Neurosci. Lett. **56**: 63-68.

Coats, S.R. and Gerard, N.P. 1989. Characterization of the substance P receptor in guinea pig lung tissues. Am. J. Respir. Cell Mol. Biol. **1**: 269-275.

Cockcroft, D.W. 1988. Airway hyperresponsiveness and late asthmatic responses. Chest **94**: 178-180.

Colebatch, H.J.H., Olsen, C.R. and Nadel, J.A. 1966. Effect of histamine, serotonin and acetylcholine on the peripheral airways. J. Appl. Physiol. **21**: 217-226.

Colebatch, H.J.H. and Engel, L.A. 1974. Constriction of the lung by histamine before and after adrenalectomy in cats. J. Appl. Physiol. **34**: 143-153.

Coleridge, H.M. and Coleridge, J.C. 1981. Afferent fibres involved in the defence reflex from the respiratory tract. Adv. Physiol. Sci. **10**: 467-477.

Coleridge, J.C. and Coleridge, H.M. 1984. Afferent vagal C fibres innervation of the lung and airways and its functional significance. Rev. Physiol. Biochem. Pharmacol. **99**: 1-110.

Cooper, J.A.D., Merrill, W.W., Buck, M.G. and Schachter, N. 1986. The relationship between bronchoalveolar recruitment and bronchoconstriction induced by a soluble extract of cotton bracts. Am. Rev. Respir. Dis. **134**: 975-982.

Crimi, N., Palmero, F., Oliveri, R., Magri, S. and Mistretta, A. 1994. Inhibition of neutral endopeptidase potentiates bronchoconstriction induced by neurokinin A in asthmatic patients. Clin. Exp. Allergy **24**: 115-120.

Cuello, A.C., Galfre, G. and Milstein, C. 1979. Detection of substance P in the central nervous system by a monoclonal antibody. Proc. Natl. Acad. Sci. **76**: 3532-3536.

Curry, J.J. 1946. The action of histamine on the respiratory tract in normal and asthmatic subjects. J. Clin. Invest. **25**: 785-791.

Daniel, E.E., Kannan, M., Davies, C. and Posey-Daniel, V. 1986. Ultrastructural studies on the neuromuscular control of human trachea and bronchial muscle. Respir. Physiol. **63**: 109-128.

Danser, A.H.J., Van-Den Ende, R., Lorenz, R.R., Flavahan, N.A. and Vanhoutte, P.M. 1987. Prejunctional β-adrenoceptors inhibit cholinergic neurotransmission in canine bronchi. J. Appl. Physiol. **62**: 785-790.

Deckers, I.A., Rampart, M., Bult, H. and Herman, A.G. 1989. Evidence for involvement of prostaglandin in modulation of acetylcholine release from canine bronchial tissue. Eur. J. Pharmacol. **167**: 415-418.

De Jongste, J.C., Mons, H., Bonta, I.L. and Kerrebijn, K.F. 1987. Non-neural components in the response of fresh human airways to electrical field stimulation. J. Appl. Physiol. **63**: 1558-1566.

Delpierre, S., Grimaud, C.H., Jammes, Y. and Mei, N. 1981. Changes in activity of vagal bronchopulmonary C-fibres by chemical and physical stimuli in the cat. J. Physiol. **316**: 61-66.

Dennis, T., Fournier, A., Cadieux, A., Pomerleau, F., Jolicoeur, F.B., St Pierre, S. and Quirion, R. 1990. hCGRP$^{8-37}$, a calcitonin gene-related peptide antagonist revealing CGRP receptor heterogeneity in brain and periphery. J. Pharmacol. Exp. Ther. **254**: 123-128.

Dennis, T., Fournier, A., St Pierre, S. and Quirion, R. 1991. Structure activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. J. Pharmacol. Exp. Ther. **251**: 718-725.

Diamond, L. and Lipscomb, W. 1970. Design of small-animal pneumotachograph. J. Appl. Physiol. **29**: 722.

Dohlman, H.C., Caron, M.G. and Lefkowitz, R.J. 1987. A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry **26**: 2657-2664.

Douglas, I.S., Dennis, M.W., Ridgway, P. and Bouhuys, A. 1972. Airway dilation and constriction in spontaneously breathing guinea pigs. J. Pharmacol. Exp. Ther. **180**: 98-109.

Dray, A. 1992a. Mechanism of action of capsaicin-like molecules on sensory neurons. Life Sci. **51**: 1759-1765.

Dray, A. 1992b. Neuropharmacological mechanisms of capsaicin and related substances. Biochem. Pharmacol. **44**: 611-615.

Dray, A., Bettaney, J. and Forster, P. 1989. Capsaicin desensitization of peripheral nociceptive fibres dose not impair sensitivity to other noxious stimuli. Neurosci. Lett. **99**: 50-54.

Drazen, J.M., Shore, S.A. and Gerard, N.P. 1989. SP-induced effects in guinea-pig lung: Effects of thiorphan and captopril. J. Appl. Physiol. **66**: 1364-1372.

Duckles, S.P., Rayner, M.D. and Nadel, J.A. 1974. Effects of CO_2 and pH on drug-induced contractions of airway smooth muscle. J. Pharmacol. Exp. Ther. **190**: 472-481 .

Ediss, C. and Biggs, D.F. 1991. A digital timing system for a small animal respiration pump. J. Pharmacol. Meth. **25**: 171-177.

Ellis, J.L. 1995. Neurokinin receptors subserving bronchoconstriction. Can. J. Physiol. Pharmacol. **73**: 923-26.

Ellis, J.L. and Farmer, S.G. 1989. Effects of peptidases on non-adrenergic non-cholinergic inhibitory responses of tracheal smooth muscle: a comparison with effects on VIP- and PHI-induced relaxation. Br. J. Pharmacol. **96**: 521-526.

Ellis, J.L. and Undem, B.J. 1992a. Antigen-induced enhancement of noncholinergic contractile responses to vagus nerve and electrical field stimulation in guinea-pigs isolated trachea. J. Pharmacol. Exp. Ther. **262**: 646-653.

Ellis, J.L. and Undem, B.J. 1992b. Inhibition by L-N^G-nitro-L-arginine of nonadrenergic noncholinergic-mediated relaxation of human isolated central and peripheral airways. Am. Rev. Respir. Dis. **146**: 1543-1547.

Emonds-Alt, X., Vilain, P. and Goulaouic, P. 1992. A potent and selective non-peptide antagonist of the neurokinin A (NK2) receptor. Life Sci. - Pharmacol. Lett. **50**: PL101-PL106.

Empey, D.W., Laitinen, L.A., Jacobs, L., Gold, W.M. and Nadel, J.A. 1976. Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infection. Am. Rev. Respir. Dis. **113**: 131-139.

Erspamer, V. 1981. The tachykinin peptide family. Trends Neurosci. **4**: 267-269.

Erspamer, V. and Melchiorri, P. 1973. Active peptides of the amphibian skin and their synthetic analogs. Pure Appl. Chem. **35**: 463-494.

Eshar, Z. 1985. Hybridoma technology in the biosciences and medicine. *In: The production and Improvement Through Genetic Engineering of Monoclonal Antibodies.* (Springer, T.A., ed.), pp: 1-41, Plenum Press, New York.

Evangelista, S., Tramontana, M. and Maggi, C.A. 1992. Pharmacological evidence for the involvement of multiple calcitonin gene-related peptide (CGRP) receptors in the antisecretory and antiulcer effect of CGRP in rat stomach. Life Sci. **50**: PL13-PL18.

Flavahan, N.A., Aarhus, L.L., Rimele, T.J. and Vanhoutte, P.M. 1985. Respiratory epithelium inhibits bronchial smooth muscle tone. J. Appl. Physiol. **58**: 834-838.

Ferreira, S.H. and Vane, J.R. 1967. Prostaglandins: their disappearance from and release into the circulation. Nature **216**: 868-873.

Fields, G.B. and Noble, R.L. 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Peptide Protein Res. **35**: 161-214.

Foreman, J. and Jordan, C.C. 1984. Neurogenic inflammation. Trends Pharmacol. Sci. **5**: 116-119.

Foulon, D.M., Champion, E., Masson, P., Rodger, I.W. and Jones, T.R. 1993. NK1 and NK2 receptors mediate tachykinin and resinferatoxin-induced bronchospasm in guinea-pigs. Am. Rev. Respir. Dis. **148**: 915-921.

Franco-Cereceda, A. 1991. Resinferatoxin-, capsaicin- and CGRP-evoked porcine coronary vasodilation is independent of EDRF mechanisms but antagonized by CGRP (8-37). Acta. Physiol. Scand. **143**: 331-337.

Franco-Cereceda, A. and Lundberg, J.M. 1985. CGRP and capsaicin-induced stimulation of heart contractile rate and force. Naunyn-Schmiedebergs Arch. Pharmacol. **331**: 146-151.

Franco-Cereceda, A., Lundberg, J.M., Saria, A., Schreibmayer, W. and Tritthart, H.A. 1988. Calcitonin gene-related peptide: release by capsaicin and prolongation of the action potential in the guinea-pig heart. Acta Physiol. Scand. **132**: 181-190.

Freezer, N.J., Lanteri, C.J. and Sly, P.D. 1993. Effect of pulmonary blood flow on measurements of respiratory mechanics using the interrupter technique. J. Appl. Physiol. **74**: 1083-1088.

Frossard, N. and Advenier, C. 1991. Tachykinin receptors and the airways. Life Sci. **49** 1941-1953.

Fryer, A.D. and Jacoby, D.B. 1991. Parainfluenza virus infection damages inhibitory M2-muscarinic receptors on pulmonary parasympathetic nerves in guinea-pig. Br. J. Pharmacol. **102**: 267-271.

Fuller, R.W. 1990. The human pharmacology of capsaicin. Arch. Int. Pharmacodyn. Ther. **303**: 147-156.

Gamse, R., Lembeck, F. and Cuello, A.C. 1979a. Substance P in the vagus nerve. Immunochemical and immunohistological evidence for axoplasmic transport. Arch. Pharmacol. **306**: 37-44.

Gamse, R., Molnar, A. and Lembeck, F. 1979b. Substance P released from spinal cord slices by capsaicin. Life Sci. **25**: 629-636.

Gamse, R. and Saria, A. 1985. Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. Eur. J. Pharmacol. **114**: 61-66.

Gaw, A.J., Aberdeen, J., Humphrey, P.P., Wadsworth, R.M. and Burnstock, G. 1991. Relaxation of sheep cerebral arteries by vasoactive intestinal polypeptide and neurogenic stimulation: inhibition by L-NG-monomethyl arginine in endothelium-denuded vessels. Br. J. Pharmacol. **102**: 567-572.

Geppetti, P, Bertrand, C., Bacci, E., Snider, R.M., Maggi, C.A. and Nadel, J.A. 1992. The NK1 receptor antagonist CP 96,345 Blocks substance P-evoked mucus secretion from the ferret trachea in vitro. Neuropeptides **22**: 25.

Gerard, N.P., Eddy, R.L., Shows, T.B. and Gerard, C. 1990. The human neurokinin A (substance K) receptor: molecular cloning of the gene, chromosome localization and isolation of cDNA from tracheal and gastric tissues. J. Biol. Chem. **265**: 20455-20462.

Gerard, N.P., Bao, L., Xiao-Ping, H. and Gerard, C. 1993. Molecular aspects of tachykinin receptors. Regul. Pept. **43**: 21-35.

Gerhardt, P., Hasenohrl, R.U. and Huston, J.P. 1992. Enhanced learning produced by injection of neurokinin substance P into the region of the nucleus basalis magnocellularis: mediation by the N-terminal sequence. Exp. Neurology. **118**: 302-308.

Giuliani, S., Wimalawansa, S. J., and Maggi, C. A. 1992. Involvement of multiple receptors in the biological effects of calcitonin gene-related peptide and amylin in rat and guinea-pig preparations. Br. J. Pharmacol. **107**: 510-514.

Goldie, R.G., Spina, D., Henry, P.J., Lulich, K.M. and Paterson, J.W. 1986. In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, β-adrenoceptor agonists and theophylline. Br. J. Pharmacol. **22**: 669-676.

Granditz, T., Ekman, R., Hakanson, R., Rerup, C., Sundler, F. and Uddman, R. 1986. Calcitonin gene-related peptide in thyroid nerve fibres and C cells: effects on thyroid hormone secretion and response to hypercalcemia. Endocrinology **119**: 2313-2324.

Green, M. and Mead, J. 1974. Time dependence of flow-volume curves. J. Appl. Physiol. **37**: 793-797.

Grundstorm, N. and Andersson, R.G.G. 1985a. Inhibition of cholinergic neurotransmission in human airways via prejunctional α_2-adrenoceptors. Acta. Physiol. Scand. **125**: 513-517.

Grundstorm, N. and Andersson, R.G.G. 1985b. In vivo demonstration of α_2-adrenoceptor mediated inhibition of the excitatory non-cholinergic neurotransmission in guinea-pigs. Naunyn-Schmiedebergs Arch. Pharmacol. **328**: 236-240.

Guard, S. and Watson, S.P. 1991. Tachykinin receptor types: classification and membrane signalling mechanisms. Neurochem. Int. **18**: 149-165.

Gulbenkian, S., Merighi, A.,Wharton, J., Varndell, I. and Polak, J.M. 1986. Ultrastructural evidence for the coexistence of CGRP and substance P in secretory vesicles of peripheral nerves in the guinea-pig. J. Neurocytol. **15**: 535-542.

Hahn, H.L. and Nadel, J.A. 1979. Methods of study of airway smooth muscle and its physiology. Pharmacol. Ther. **7**: 253-295.

Hall, M.E. and Stewart, J.M. 1990. The substance P fragment SP^{7-11} stimulates motor behaviour and nigral dopamine release. Pharmacol. Biochem. Behaviour **41**: 75-78.

Hamel, R. and Ford-Hutchinson, A.W. 1988. Contractile activity of calcitonin gene-related peptide on pulmonary tissues. J. Pharm. Pharmacol. **40**: 210-211.

Hanley, M.R., Lee, C.M., Jones, L.M. and Michell, R.H. 1980. Similar effects of substance P and related peptides on salivation and on phosphatidylinositol turnover in rat salivary glands. Mol. Pharmacol. **18**: 78-83.

Hargreave, F.E., Ryan, G., Thomson, N.C., O'Byrne, P.M., Latimer, K., Juniper, E.F. and Dolavich, J. 1981. Bronchial responsiveness to histamine or methacholine in asthma: Measurement and clinical significance. J. Allergy Clin. Immunol. **68**: 347-355.

Heifetz, A.H., Braats, J.A., Wolfe, R.A., Barry, R.M., Miller, D.A. and Solomon, B.A. 1989. Monoclonal antibody production in hollow fibre bioreactors using serum-free medium. Biotechniques **7**: 192-199.

Hermansen, K. and Ahren, B. 1990. Dual effects of calcitonin gene-related peptide on insulin secretion in the perfused dog pancreas. Regul. Pept. **27**: 147-149.

Hershey, A.D. and Cruse, J.E. 1990. Molecular characterization of a functional cDNA encoding the rat substance P receptor. Science **247**: 958-962.

Higgs, G.A., Copp, F.C., Denyer, C.V., Flower, R.J., Tateson, J.E., Vane, J.R. and Walker, J.M.G. 1978. Reduction of leucocyte migration by a cyclo-oxygenase and lipoxygenase inhibitor. *Proceeding of the Seventh International Congress of Pharmacology*. Abstr. 843, P. 33. Oxford, Pergamon Press.

Hogman, M., Frostell, C.G., Hedenstrom, H. and Hedenstierna, G. 1993. Inhalation of nitric oxide modulates adult human bronchial tone. Am. Rev. Respir. Dis. **148**: 1474-1478.

Hiroki, O. and Shigetada, N. 1991. Molecular characterization of the three tachykinin receptors. Annu. N. Y. Acad. Sci. **632**: 53-62.

Hoshiko, K., Kristersson, A. and Morley, J. 1993. Ketotifen inhibits exacerbation of allergic airway hyperreactivity by racemic salbutamol in the guinea pig. J. Allergy Clin. Immunol. **91**: 909-916.

Hoshiko, K. and Morley, J. 1993. Exacerbation of airway hyperreactivity by racemic salbutamol in sensitized guinea-pigs. Jap. J. Pharmacol. **63**: 159-163.

Hua, X.Y., Kin, A.C. and Lundberg, J.M. 1986. Capsaicin-sensitive nerves and ureteric motility: opposing effects of tachykinins and calcitonin gene-related peptide. Acta. Physiol. Scand. **128**: 317-318.

Hua, X.Y. and Lundberg, J.M. 1986. Dual capsaicin effects on ureteric motility: low dose inhibition mediated by CGRP and high dose stimulation by tachykinins? Acta. Physiol. Scand. **128**: 453-465.

Hunter, J.C., Geodert, M. and Pinnock, R.D. 1985. Mammalian tachykinin-induced hydrolysis of inositol phospholipid in rat brain slices. Biochem. Biophys. Res. Commun. **127**: 616-622.

Ichinose, M., Miura, M., Takahashi, T., Yamauchi, H., Nakajima, M., Tomaki, M., Inoue, H., Takishima, T. and Shirato, K. 1993. Neurogenic inflammation. Jap. J. Thoracic Dis. **31 Suppl**: 172-175.

Ichinose, M., Nakajima, N. and Takahashi, T. 1992. Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. Lancet **340**: 1248-1251.

Ichinose, M., Stretton, C.D., Schwartz, J.C. and Barnes, P.J. 1989. Histamine H_3-receptors inhibit cholinergic neurotransmission in guinea-pig airways. Br. J. Pharmacol. **97**: 13-15.

Inoue, T. and Ito, Y. 1986. Characteristics of neuro-effector transmission in the smooth muscle layer of dog bronchioles and modification by autocoids. J. Physiol. **370**: 551-556.

Irvin, C.G., Martin, R.R. and Macklem, P.T. 1982. Nonpurinergic nature and efficacy of nonadrenergic bronchodilation. J. Appl. Physiol. **52**: 562-569.

Jafarian, A., Handley, D.A. and Biggs, D.F. 1996. Effects of RS-albuterol on the development of antigen-mediated airway hyperreactivity in guinea-pigs. Clin. Rev. Allergy Immunol. In press.

Jafarian, A., Suresh, M.R., Kreutz, F.T. and Biggs, D.F. 1995. Passive immunization with an anti-substance P antibody prevents substance P- and neurokinin A-induced bronchospasm in anesthetized guinea-pigs. Life Sci. **57**: 143-153.

Jansco, N., Jansco-Gabor, A. and Szolcsanyi, J. 1986. The role of sensory nerve endings in neurogenic inflammation induced in human skin and the eye and paw of rat. Br. J. Pharmacol. **32**: 32-41.

Jansco, N., Karcsu, S., Kiraly, E., Szebeni, A., Toth, L., Bacsy, E., Joo, F. and Parducz, A. 1984. Neurotoxin-induced cell degeneration: possible involvement of calcium. Brain Res. **295**: 211-216.

James, J.B. and Gray, W.W. 1992. Calcitonin gene-related peptide potentiates substance P-induced plasma extravasation in the rat trachea. **170**: 85-93.

James, A.L., Pare, P.D., Moreno, R. and Hogg, J.L. 1986. Smooth muscle shortening and airway narrowing in porcine tracheal rings. Am. Rev. Respir. Dis. **133**: A113.

James, W., Puteny, J.R. and Haruo, T. 1991. Role of inositol phosphates in the action of substance P on NK1 receptor in exocrine gland cells. Annu. N. Y. Acad. Sci. **632**: 94-102.

Jancso, G., Karcsu, S., Kiraly, E., Szebeni, A., Toth, L., Bacsy, E., Joo, F. and Parducz, A. 1984. Neurotoxin-induced nerve cell degeneration: possible involvement of calcium. Brain Res. **295**: 211-216.

Johnson, C.L., Johnson, C.G., Stauderman, K.A. and Bucks, S.H. 1991. Characterization of substance P receptor in human astrocytoma cells. Annu. N. Y. Acad. Sci. **632**: 410-412.

Joos, G.F., Germonpre, P.R., Kips, J.C., Peleman, R.A. and Pauwels, R.A. 1994. Sensory neuropeptides and human lower airways: present state and future direction. Eur. Respir. J. **7**: 1161-1167.

Joos, G.F. and Pauwels, R.A. 1990. Mechanisms involved in neurokinin-induced bronchoconstriction. Arch. Int. Pharmacodyn. Ther. **303**: 132-46.

Kannan, M.S. and Johnson, D.E. 1992. Nitric oxide mediates the neural non-adrenergic, non-cholinergic relaxation of pig tracheal smooth muscle. Am. J. Physiol. **262**: L511-L514.

Karlsson, J.A. and Persson, C.G.A. 1985. Effect of different substance P analogues on tachykinin-induced contraction of airway smooth muscle. *In: Tachykinin Antagonists*, (Hakanson, R. and Sundler, F., eds.), pp: 181-185, Amsterdam: Elsevier.

Karlsson, J.A. and Persson, C.G.A. 1989. Novel peripheral neurotransmitters and control of the airways. Pharmacol. Ther. **43**: 397-423.

Knazek, R.A., Gullino, P.M., Kohler, P.O. and Dedrick, R.L. 1972. Cell culture on artificial capillaries: an approach to tissue growth in vitro. Science **178**: 65-67.

Knazek, R.A., Wu, Y-W., Aebersold, P.M. and Rosenberg, S.A. 1990. Culture of human tumour infiltrating lymphocytes in hollow fibre reactors. J. Immunol. Meth. **127**: 29-37.

Kohler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256**: 495-497.

Kohrogi, H., Graf, P.D., Sekizqawa, K., Borson, D.B. and Nadel, J.A. 1988. Neutral endopeptidase inhibitors potentiate substance P- and capsaicin-induced cough in awake guinea-pigs. J. Clin. Invest. **82**: 2063-2068.

Krause, J.E., Yasuo, T. and Andrew, D.H. 1992. Structure, function and mechanism of substance P receptor action. J. Invest. Dermatol. **98**: 2S-7S.

Kroll, F., Karlsson, J.A., Lundberg, J.M., and Persson, C.G.A. 1990. Capsaicin-induced bronchoconstriction and neuropeptide release in guinea-pig perfused lungs. J. Appl. Physiol. **68**: 1679-1687.

Kudlacz, E.M. and Knippenberg, R.W. 1994. In vitro and in vivo effects of tachykinins on immune cell function in guinea-pig airways. J. Neuroimmunol. **50**: 119-125.

Kume, H., Graziano, M.P. and Kotlikoff, M.I. 1992. Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. Proc. Natl. Acad. Sci. USA **89**: 11051-11055.

Kummer, W., Fischer, A., Mundel, P., Mayer, B., Hoba. B., Philippin, B. and Preissler, U. 1992. Nitric oxide synthase in VIP-containing vasodilator nerve fibres in the guinea-pig. Neuro. Report. **3**: 653-655.

Kundu, G.C. and Wilson, I.B. 1992. Identification of endothelin-converting enzyme in bovine lung membranes using a new fluorogenic substrate. Life Sci. **50**: 965-970.

Kuo, H.P., Liu, S. and Barnes, P.J. 1992. The effect of endogenous nitric oxide on neurogenic plasma exudation in guinea-pig airways. Eur. J. Pharmacol. **221**: 385-388.

Kuo, H.P., Rohde, J.A.L. and Rogers, D.F. 1990. Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea-pig tracheal. J. Physiol. **431**: 629-641.

Ladenius, A.R.C., Campbell, J.W., Sivaprasad, S., Suresh, M. and Biggs, D.F. 1991. Active immunization against substance P prevents the development of airway hyperresponsiveness in a guinea-pig model of asthma. Am. Rev. Respir. Dis. **143 Suppl.** A614.

Ladenius, A.R.C. 1993. Ph.D.Thesis, University of Alberta.

Laitinen, L.A., Heino, M., Laitinen, A., Kava, T. and Haahtela, T. 1985. Damage of the epithelium and bronchial reactivity in patients with asthma. Am. Rev. Respir. Dis. **131**: 599-606.

Lammers, J-W.J., Barnes, P.J. and Chung, K.F. 1992. Nonadrenergic, noncholinergic airway inhibitory nerves. Eur. Respir. J. **5**: 239-248.

Larson, A.A. and Sun, X. 1994. Role of the NH2-terminus of substance P in the inhibition by capsaicin behavioural sensitization to kainic acid-induced activity in the adult mouse. J. Pharmacol. Exp. Ther. **268**: 366-371.

Laufer, R., Gilon, C., Chorev, M. and Selinger, J. 1986. Characterization of a neurokinin B receptor site in rat brain using a highly selective radioligand. J. Biol. Chem. **261**: 10257-10263.

Lazarus, S.C., Basbaum, C.B., Barnes, P.J. and Gold, W.M. 1986. cAMP immunocytochemistry provides evidence for functional VIP receptors in trachea. Am. J. Physiol. **251**: C115-C119.

Lee, C.M., Javitch, J.A. and Synder, S.H. 1983. ^{3}H-substance P binding to salivary gland membranes. Mol. Pharmacol. **23**: 653-669.

Leff, A.R. 1988. State of the art. Endogenous regulation of bronchomotor tone. Am. Rev. Respir. Dis. **137**: 1198-1216.

Leff, A.R. and Munoz, N.M. 1980. Interrelationship between alpha- and beta-adrenergic agonists and histamine in canine airways. J. Allergy. Clin. Immunol. **68**: 300-309.

Leff, A.R. and Munoz, N.M. 1981. Cholinergic and alpha adrenergic augmentation of histamine-induced contraction of canine airway smooth muscle. J. Pharmacol. Exp. Ther. **218**: 582-587.

Leff, A. and Munoz, N.M. 1981b. Selective autonomic stimulation of canine tracheal with dimethylphenylpiperazinium. J. Appl. Physiol. **51**: 528-537.

LeGreves, P., Hyberg, F., Hokfelt, T and Terenius, L. 1989. Calcitonin gene-related peptide is metabolized by an endopeptidase hydrolysing substance P. Regul. Pept. **25**: 277-286.

Lei, Y.H., Barnes, P.J. and Rogers, D.F. 1993. Regulation of NANC neural bronchoconstriction in vivo in the guinea-pigs: involvement of nitric oxide, vasoactive intestinal peptide and soluble guanylyl cyclase. Br. J. Pharmacol. **108**: 228-235.

Lihme, A. and Heegaard, P.M.H. 1990. Thiophilic adsorption chromatography: the separation of serum proteins. Annu. Biochem. **192**: 64-69.

Lilly, C.M., Besson, G., Isreal, E., Rodgers, I.W. and Drazen, J.M. 1994. Capsaicin-induced airway obstruction in tracheally perfused guinea-pig lungs. Am. J. Respir. Crit. Care. Med. **149**: 1175-1179.

Lilly, C.M., Drazen, J.M. and Shore, S.A. 1993. Peptidase modulation of airway effects of neuropeptides. Proc. Soc. Exp. Biol. Med. **203**: 388-404.

Longmore, J., Razzaque, D. and Hill, R.G. 1994. Differences in the effects of NK1 receptor antagonists, (±)-CP 96345 and CP 99994, on agonist-induced responses in guinea-pig trachea. Br. J. Pharmacol. **112**: 176-178.

Lou, Y.P., Franco-Cereceda, A. and Lundberg, J.M. 1991. Omega-conotoxin inhibits CGRP release and bronchoconstriction evoked by low concentration of capsaicin. Acta. Physiol. Scand. **141**: 135-136.

Lundberg, J.M. 1993. New aspects of airway innervation. Pharmacol. Toxicol. **72 Suppl 3**: 21-27.

Lundberg, J.M. 1995. Tachykinin, sensory nerves and asthma - an overview. Can. J. Physiol. Pharmacol. **73**: 908-914.

Lundberg, J.M., Brodin, E. and Saria, A. 1983a. Effect and distribution of vagal capsaicin-sensitive substance P neurons with special reference to the trachea and lung. Acta. Physiol. Scand. **119**: 243-259.

Lundberg, J.M., Franco-Cereceda, A., Alving, K., Delay-Goyet, P. and Lou, Y. P. 1992. Release of calcitonin gene-related peptide from sensory neurons. Annu. N. Y. Acad. Sci. **657**: 187-193.

Lundberg, J.M., Franco-Cereceda, A., Hua, X.Y., Hokfelt, T. and Fischer, J.A. 1985. Coexistence of substance P and calcitonin gene-related peptide immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. Eur. J. Pharmacol. **108**: 315-319.

Lundberg, J.M., Lundblad, L., Martling, C.R., Saria, A., Stjarne, P. and Anggard, A. 1987. Co-existence of multiple peptides and classical transmitters in airway neurons: functional and pathophysiological aspects. Am. Rev. Respir. Dis. **136**: S16-S22.

Lundberg, J.M. and Saria, A. 1987. Polypeptide containing neurons in airway smooth muscle. Annu. Rev. Physiol. **49**: 557-572.

Lundberg, J.M., Saria, A., Brodin, E., Rogeli, S. and Folkers, K. 1983b. A substance P antagonist inhibits vagally-induced increase in vascular permeability and bronchial smooth muscle contraction in guinea-pig. Proc. Natl. Acad. Sci. U.S.A. **80**: 1120-1124.

Luts, A., Widmark, E., Ekman, R., Waldeck, B. and Sundler, F. 1990. Neuropeptides in guinea-pig trachea: distribution and evidence for the release of CGRP into tracheal lumen. Peptides. **11**: 1211-1216.

Maclagan, J. and Ney, U.M. 1979. Investigation of the mechanism of propranolol-induced bronchoconstriction. Br. J. Pharmacol. **66**: 409-418.

Maggi, C.A. 1990. Tachykinin receptors in the airways and lung: what should we block? Pharmacol. Res. **22**: 527-540.

Maggi, C.A. 1991. Capsaicin and primary afferent neurons: from basic science to human therapy? J. Auton. Nerv. System **33**: 1-14.

Maggi, C.A. 1993. Tachykinin receptors and airway pathophysiology. Eur. Respir. J. **6**: 735-742.

Maggi, C.A., Astolfi, M., Donneree, J. and Amann, R. 1990a. Which mechanism accounts for the sensory neuron blocking action of capsaicin on primary afferent in the rat urinary bladder? Neurosci. Lett. **110**: 267-272.

Maggi, C.A., Giuliani, S., Ballati, L., Lecci, A., Manzini, S., Patacchini, R., Renzetti, A.R., Rovero, P., Quartara, L. and Giachetti, A. 1991c. In vivo evidence for tachykininergic transmission using a new NK-2 receptor-selective antagonist, MEN 10,376. J. Pharmacol. Exp. Ther. **257**: 1172-1178.

Maggi, C.A., Giuliani, S., Manzini, S., and Meli, A. 1989a. $GABA_A$ receptor-mediated positive ionotropism in guinea-pigs isolated left atria: evidence for the involvement of capsaicin sensitive nerves. Br. J. Pharmacol. **97**: 103-110.

Maggi, C.A. and Meli, A. 1988. The sensory-efferent function of capsaicin-sensitive sensory neurons. Gen. Pharmacol. **19**: 1-43.

Maggi, C.A., Patacchini, R. and Astolfi, M. 1991a. NK2 receptor agonists and antagonists. Annu. N. Y. Acad. Sci. **632**: 184-191.

Maggi, C.A., Patachini, R., Rovero, P. and Giachetti, A. 1993. Tachykinin receptors and receptor antagonists. J. Auton. Pharmacol. **13**: 1-75.

Maggi, C.A., Patachini, R., Santicioli, P., Lippe, I.T., Giuliani, S., Geppetti, P., Del Binaco, E., Seleri, S. and Meli, A. 1988b. The effect of omega conotoxin GVIA, a peptide modulator of N-type voltage-sensitive calcium channels on motor response produced by activation of efferent and sensory nerves in mammalian smooth muscle. Naunym Schmiedeberg's Arch. Pharmacol. **338**: 107-113.

Maggi, C.A., Rovero, P., Giuliani, S., Evangelista, S., Regoli, D. and Meli, A. 1990c. Biological activities of N-terminal fragments of calcitonin gene-related peptide. Eur. J. Pharmacol. **179**: 217-219.

Maggi, C.A., Santicioli, P., Abeli, L., Parlani, M., Capasso, M., Conte, B., Guliani, S. and Meli, A. 1987a. Regional differences in the effect of capsaicin and tachykinins on motor activity and vascular permeability in the rat lower urinary tract. Naunyn-Schmiedebergs Arch. Pharmacol. **335**: 636-645.

Maggi, C.A., Santicioli, P., Geppetti, P., Furio, M., Frilli, S., Conto, B., Fanciullacci, M. Giuliani, S. and Meli, A. 1987b. The contribution of capsaicin-sensitive innervation to activation of the spinal vesico-vesical reflex in rat, relationship between substance P level in the urinary bladder and the sensory-efferent function of capsaicin-sensitive sensory neurons. Brain Res. **415**: 1-13.

Maggi, C.A., Santicioli, P., Geppetti, P., Parlani, M., Astolfi, M. and Del Bianco, E. 1989b. The effect of calcium free medium and nifedipine on the release of substance P-like immunoreactivity and contraction induced by capsaicin in the isolated guinea-pig and rat bladder. Gen. Pharmacol. **20**: 445-456.

Maggi, C.A., Santicioli, P. and Meli, A. 1985. Evidence for the involvement of endogenous substance P in the motor effect of capsaicin on the rat urinary bladder. J. Pharm. Pharmacol. **37**: 203-204.

Maggio, J.E. 1985. "Kassinin" in mammals: the newest tachykinin. Peptides **6 Suppl**: 237-245.

Maggio, J.E. 1988. Tachykinins. Annu. Rev. Neurosci. **11**: 13-28.

Mak, J.W.C. and Barnes, P.J. 1989. Muscarinic receptor subtypes in human and guinea-pig lung. Eur. J. Pharmacol. **164**: 223-230.

Mantyh, P.W., Pinnock R.D., Dowens, C.P., Goedert, M. and Hungs, S.P. 1984. Nature **309**: 795-797.

Manzini, S., Conti, S., Maggi, C.A., Abelli, L., Somma, V., Del Binaco, E. and Geppetti, T. 1989. Regional differences in the motor and inflammatory responses to capsaicin in guinea-pig airways. Am. Rev. Respir. Dis. **140**: 936-941.

Manzini, S., Maggi, C.A., Gepetti, P. and Bacciarelli, C. 1987. Capsaicin desensitization protects from antigen-induced bronchospasm in conscious guinea-pigs. Eur. J. Pharmacol. **138**: 307-308.

Mapp, C., Chitano, P., Fabri, L., Patacchini, R. and Maggi, C.A. 1990. Pharmacological modulation of contractile response to toluene diisocyanate in the rat isolated urinary bladder. Br. J. Pharmacol. **100**: 886-888.

Marsh, S.J., Stansfeld, C.E., Brown, D.A., Davey, R. and McCarthy, D. 1988. The mechanism of action of capsaicin on sensory C-type neurons and their axon in vitro. Neurosci. **23**: 275-289.

Martin, C.A., Naline, E., Emonds, Alt-X. and Advenier, C. 1992. Influence of (+/-)-CP 96,345 and SR 48,968 on electrical field stimulation of the isolated guinea-pig main bronchus. Eur. J. Pharmacol. **224**: 137-143.

Martin, J.G. 1994. Animal models of bronchial hyperreactivity. Reveu. des Maladies Respir. **11**: 93-99.

Martling, C.R., Martran, R., Alving, K., Hokfelt, T. and Lundberg, J.M. 1990. Innervation of the lower airways and neuropeptide effects on bronchial and vascular tone in the pig. Cell Tissue Res. **260**: 223-233.

Martling, C.R., Saria, A., Fisher, J.A., Hokfelt, T. and Lundberg, J.M. 1988. Calcitonin gene-related peptide and the lung: neuronal coexistence with substance P, release by capsaicin and vasodilatory effect. Regul. Pept. **20**: 125-139.

Masata, R., Kenny, A.J. and Turner, A.J. 1984. The metabolism of neuropeptides: the hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. Biochem. J. **223**: 433-440.

Matran, R., Martling, C.R. and Lundberg, J.M. 1989. Inhibition of cholinergic and non-adrenergic, non-cholinergic bronchoconstriction in the guinea-pig mediated by neuropeptide Y and α_2-adrenoceptors and opiate receptors. Eur. J. Pharmacol. **163**: 15-23.

Matsumoto, N., Inoue, H., Ichinose, M., Ishii, M., Inoue, C., Sasaki, H. and Takishima, T. 1985. Effective sites by sympathetic β-adrenergic and vagal nonadrenergic inhibitory stimulation in constricted airways. Am. Rev. Respir. Dis. **132**: 1113-1117.

Matsuyama, T., Wanaka, A., Yoneda, S., Kimura, K., Kamada, T., Girgis, S., MacIntyre, I., Emson, P.C. and Tohyama, M. 1986. Two distinct CGRP-containing peripheral nervous system: distribution and quantitative differences between the iris and cerebral artery with special reference to substance P. Brain Res. **373**: 205-212.

Matsuzuki, Y., Hamasaki, Y. and Said, S.I. 1980. Vasoactive intestinal peptide: a possible transmitter of nonadrenergic relaxation of guinea-pig airways. Science **210**: 1252-1253.

Mazzoni, L., Naef, R., Chapman, I.D. and Morley, J. 1995. Hyperresponsiveness of the airways following exposure of guinea-pigs to racemic mixtures and distomers of β_2-selective sympathomimetics. Pulmonary Pharmacol. **8**: in press.

McCaig, D.J. 1987. Effects of sympathetic stimulation and applied cathecolamines on mechanical and electrical responses to stimulation of the vagus nerve in guinea-pig isolated trachea. Br. J. Pharmacol. **91**: 385-394.

McCulloch, J., Uddman, R., Kingman, T.A. and Edvinsson, L. 1986. Calcitonin gene-related peptide: functional role in the cerebrovascular regulation. Proc. Natn. Acad. Sci. **83**: 5731-5735.

McDonald, D.M., Schoeb, T.R. and Lindsey, J.R. 1991. Mycoplasma pulmonis infections cause long-lasting potentiation of neurogenic inflammation in the respiratory tract of the rat. J. Clin. Invest. **87**: 787-799.

McDonald, D.M. 1992. Infections intensify neurogenic plasma extravasation in the airway mucosa. Am. Rev. Respir. Dis. **146 Suppl.**: S40-S44.

McDonald, D.M. 1994. Endothelial gaps and permeability of venules in rat trachea exposed to inflammatory stimuli. Am. J. Physiol. **266**: L61-L83.

McNeill, R.S. 1987. Effect of propranolol on ventilatory function. Am. J. Cardiol. **18**: 473-475.

Michoud, M.C., Amoyt, R., Jeanneret-Grossjean, A. and Couture, J. 1987. Reflex decrease of histamine-induced bronchoconstriction after laryngeal stimulation in humans. Am. Rev. Respir. Dis. **136**: 618-622.

Miles, K., Greengard, P. and Huganir, R.L. 1989. Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes. Neurons **2**: 1517-1524.

Mimeault, M., Fournier, A., Dumount, Y., St-Pierre, S. and Quirion, R. 1991. Comparative affinities and antagonistic potencies of various human calcitonin gene-related peptide fragments on calcitonin gene-related peptide receptors in brain and periphery. J. Pharmacol. Exp. Ther. **258**: 1084-1090.

Minette, P.A.H., Lammers, J., Dixon, C.M.S. and McCusker, P.J. 1989. A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. J. Appl. Physiol. **67**: 2461-2465.

Mitchell, E.A. 1989. Is current treatment increasing asthma mortality and morbidity? Thorax **44**: 81-84.

Mitzner, W., Blosser, S., Yager, D. and Wagner, E. 1992. Effect of bronchial smooth muscle contraction on lung compliance. J. Appl. Physiol. **72**: 158-167.

Molnar, J., Makara, G., Gyorgy, L. and Unyi, G. 1969. The bronchoconstrictor action of capsaicin in the guinea-pig. Acta Physiologica Academiae Scientiarum Hungarica **36**: 413-420.

Morley, J. 1994. Parasympatholytics in asthma. Pulmonary Pharmacol. **7**: 159-168.

Morley, J., Chapman, I.D., Foster, A., Hoshiko, K. and Mazzoni, L. 1991. Effects of (+) and racemic salbutamol on airway responses in the guinea-pig. Br. J. Pharmacol. **104**: 295P.

Morris , H.R., Panico, M., Etienne, T., Tippins, J., Girgis, S.I. and MacIntyre, I. 1984. Isolation and characterization of calcitonin gene-related peptide. Nature **308**: 746-748.

Mousli, M., Bronner, C., Bube, J.L., Tschirhart, E., Gies, J.P. and Landry, Y. Activation of rat peritoneal mast cells by substance P and mastoparan. 1989. J. Pharmacol. Exp. Ther. **250**: 329-335.

Mousli, M., Bueb, J.L., Bronner, C., Rouot, B. and Landry, Y. 1990. G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom. Trends Pharmacol. Sci. **11**: 358-362.

Nadel, J.A. 1965. Structure-function relationship in the airways: Bronchoconstriction mediated via vagus nerves or bronchial arteries; peripheral lung constriction mediated via pulmonary arteries. Med. Thorac. **22**: 231-242.

Nadel, J.A. 1977. Autonomic control of airway smooth muscle and airway secretions. Am. Rev. Respir. Dis. **115**: 117-126.

Nadel, J.A. 1980. Autonomic regulation of airway smooth muscle. *In: Physiology and Pharmacology of the Airways.* (Nadel, J.A. ed.) pp: 217-257, Marcel Dekker Inc., New York.

Nadel, J.A. and Barnes, P.J. 1984. Autonomic regulation of the airways. Annu. Rev. Med. **35**: 451-467.

Nadel, J.A., Cabezas, G.A. and Austin, J.H.M. 1971. In vivo roentgenographic examination of parasympathetic innervation of small airways. Invest. Radiol. **6**: 9-17.

Nadel, J.A., Colebatch, H.I.H. and Olsen, C.R. 1964. Location and mechanism of airway constriction after barium sulphate microembolism. J. Appl. Physiol. **19**: 387-394.

Nadel, J.A., Corn, M., Zwi, S., Fleisch, I. and Graf, P. 1966. Location and mechanism of airway constriction after inhalation of histamine aerosol and inorganic sulphate aerosol. *In: Inhaled Particles and Vapours* (British Occupational Hygiene Society, ed.) pp: 55-67 Oxford & New York: Pergamon Press.

Nadel, J.A. and Tierney, D.F. 1961. Effect of a previous deep inspiration on airway resistance in man. J. Appl. Physiol. **16**: 717-719.

Nadel, J.A., Wolfe, W.G. and Graf, P.D. 1968. Powdered tantalum as a medium for bronchography in canine and human lungs. Invest. Radiol. **3**: 229-238.

Nakamuta, N., Fukuda, Y. and Koida, M. 1986. Binding site of calcitonin gene-related peptide: abundant occurrence in visceral organ. Jap. J. Pharmacol. **42**: 175-180.

Nakanishi, S. 1991. Mammalian tachykinin receptors. Annu. Rev. Neurosci. **14**: 123-136.

National Heart, Lung and Blood Institute. 1991. Asthma education program expert panel report: guidelines for the diagnostic and management of asthma. Bethesda, National Institute of Health Publication No. 91-3042.

Nelly, F. and Charles, A. 1991. Tachykinin receptors and the airways. Life Sci. **49**: 1941-1953.

Ney, U.M. 1983. Propranolol-induced airway hyperreactivity in guinea-pigs. Br. J. Pharmacol. **79**: 1003-1009.

Nicolau, M., Sirois, M.G., Bui, M., Plante, G.E., Sirois, P. and Regoli, D. 1993. Plasma extravasation induced by neurokinins in conscious rats: receptor characterization with agonists and antagonists. Can. J. Physiol. Pharmacol. **71**: 217-721.

Ohhashi, T. and Jacobowitz, D.M. 1985. Effect of calcitonin gene-related peptide on the neuroeffector mechanism of sympathetic nerve terminals in rat vas deferens. Peptides 6: 987-991.

Okimura, Y, Chihara, K., Abe, H., Kaji, H., Kita, T., Kaschio, Y. and Fujita, T. 1986. Effect of intracerebroventricular administration of rat calcitonin gene-related peptide (CGRP), human calcitonin and [Asu1,7]-eel calcitonin on gastric acid secretion in rats. Jap. J. Pharmacol. **33**: 273-277.

O'Neill, T.P. 1991. Mechanism of capsaicin action: recent learning. Respir. Med. **85 Suppl. A**: 35-41.

Opgenorth, T.J., Wu-Wong, J.R. and Shiosaki, A. 1992. Endothelin-converting enzymes. FASEB J. **6**: 2653-2659.

Oscarsson, S., Chaga, G. and Porath, J. 1991. Thiophilic adsorbents for RIA and ELISA procedures. J. Immunol. Meth. **143**: 143-149.

Palmer, J.B. and Barnes, P.J. 1987. Neuropeptides and airway smooth muscle function. Am. Rev. Respir. Dis. **136**: 550-554.

Palmer, J.B., Cuss, F.M.C., Ghatei, M.A.,Springal, D.R., Cadieux, A., Bloom, S.R., Polak, J.M. and Barnes, P.J. 1987a. Calcitonin gene-related peptide is localized in human airways nerves and potently constricts human airway smooth muscle. Br. J. Pharmacol. **91**: 95-101.

Palmer, R.M.J., Ferrige, A.J. and Moncada, S.A. 1987b. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature **327**: 524-527.

Parsons, G.H., Nicol, G.M., Barnes, P.J. and Chung, K.F. 1992. Peptide mediator effects on bronchial blood velocity and lung resistance in conscious sheep. J. Appl. Physiol. **72**: 1118-1122.

Patel, K.R. and Kerr, J.W. 1975. Effect of alpha receptor blocking drug, thymoxamine, on allergen induced bronchoconstriction in extrinsic asthma. Clin. Allergy **5**: 311-316.

Payan, D.G., Brewster, D.R. and Goetzl, E.J. 1984. Stereospecific receptors for substance P on cultured human IM-9 lymphoblasts. J. Immunol. **133**: 3260-3265.

Payan, D.G, McGillis, J.P. and Organist, M.L. 1986. Binding characteristics and affinity labelling of protein constituents of the human IM9 lymphoblast receptor for substance P. J. Biol. Chem. **261**: 14321-14329.

Pendry, Y.D. 1993. Neural control of airways smooth muscle. Pharmacol. Ther. **57**: 171-202.

Pendry, Y.D. and McLagan, J. 1991. Evidence for inhibition of sympathetic neuro-transmission by endogenously released acetylcholine in guinea-pig trachea. Br. J. Phamac. **104**: 817-822.

Pernow, B. 1983. Substance P. Pharmacol. Rev. **35**: 85-141.

Picard, P., Boucher, S. and Couture, R. 1993. Use of non-peptide tachykinin receptor antagonists to substantiate the involvement of NK1 and NK2 receptors in a spinal nociceptive reflex in the rat. Eur. J. Pharmacol. **132**: 255-261.

Pintal, A.S. 1986. The visceral sensations: some basic mechanisms. Prog. Brain Res. **67**: 3-19.

Piper, P.J. and Vane, J.R. 1971. The release of prostaglandins from lung and other tissues. Annu. N. Y. Acad. Sci. **180**: 363-385.

Ploy-Song-Sand, Y., Corbin, R.P. and Engel, L.A. 1978. Effects of intravenous histamine on lung mechanics in man after beta-blockade. J. Appl. Physiol. **44**: 690-695.

Poch, G. and Holzman, S. 1980. Quantitative estimation of overadditive and underadditive drug effects means of theoretical additive dose-response curves. J. Pharmacol. Meth. **4**: 179-188.

Polak, J.M. and Bloom, S.R. 1986. Regulatory peptides of the gastrointestinal and respiratory tracts. Arch. Int. Pharmacodyn. Ther. **280**: 16-49.

Porath, J., Maisano, F. and Belew, M. 1985. Thiophilic adsorption - a new method for protein fractionation. FEBS Lett. **185**: 306-310.

Pouradier Duteil, X., Sene, C. and Boschetti, E. 1990. Choice of culture medium and purification process applied to monoclonal antibodies devote to in vivo use. *In: Developments in biological standardization monoclonal antibodies for therapy, prevention and in vivo diagnosis of human disease* (Van de Donk, H. and Hennessen, W. eds.), Vol. 71, pp: 79-85, Karger, Basel.

Pretolani, M., Lefort, J. and Vargaftig, B.B. 1989. Limited interference of specific PAF antagonists with hyperresponsiveness to PAF itself of lungs from actively sensitized guinea-pigs. Br. J. Pharmacol. **97**: 433-442.

Qian, Y. and Emonds, Alt-X. and Advenier, C. 1993. Effect of capsaicin, (+/-)-CP 96,345 and SR 48,968 on the bradykinin-induced airways microvascular leakage in guinea-pigs. Pulmonary Pharmacol. **6**: 63-67.

Quirion, R., Dam, T.V. and Guard, S. 1991. Selective neurokinin receptor radioligands. Annu. N. Y. Acad. Sci. **632**: 137-44.

Quirion, R., Rossum, D.V., Dumont, Y., St-Pierre, S. and Fournier, A. 1992. Characterization of $CGRP_1$ and $CGRP_2$ receptor subtypes. Annu. N. Y. Acad. Sci. **657**: 88-105.

Rand, M.J. 1992. Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuroeffector transmission. Clin. Exp. Pharmacol. Physiol. **19**: 147-169.

Rath, S., Stanley, C.M. and Steward, M.W. 1988. An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. J. Immunol. Meth. **106**: 245-249.

Regoli, D., Escher, E., D'Orleans-Juste, P., Drapeau, G.D. and Mizrahi, J. 1984a. Receptors for substance P. III. Classification by competitive antagonists. Eur. J. Pharmacol. **97**: 171-189.

Regoli, D., Escher, E. and Mizrahi, J. 1984b. Substance P - structure-activity studies and the development of antagonists. Pharmacology **28**: 301-320.

Regoli, D., Rhaleb, N.E., Dion, S., Tousignant C., Rouissi, N., Jukic, D. and Drapeau, G. 1990. Neurokinin A: A pharmacological study. Pharmacol. Res. **22**: 1-14.

Regoli, D., Nantel, F., Tousignant, C., Jukic, D., Rouissi, N., Rhaleb, N.E., Telemaquoe, S., Drapeau, G. and D'Orleans-Juste, P. 1991. Neurokinin agonists and antagonists. Annu. N. Y. Acad. Sci. **632**: 170-183.

Ren, J., Young, R.L., Lassiter, D.C., Rings, M.C. and Harty, R.F. 1992. Calcitonin gene-related peptide: mechanisms of modulation of antral endocrine cells and cholinergic neurons. Am. J. Physiol. **62**: G732-G739.

Rhoden, K.J. and Barnes, P.J. 1990. Potentiation of non-adrenergic neural relaxation in guinea-pigs airways by a cyclic AMP phosphodiesterase inhibitor. J. Pharmacol. Exp. Ther. **252**: 396-402.

Rhoden, K.J., Meldrum, L.A. and Barnes, P.J. 1988. Inhibition of cholinergic neurotransmission in human airways by β-adrenoceptors. J. Appl. Physiol. **65**: 700-705.

Richardson, J.B. 1979. State of the art. Nerve supply the lungs. Am. Rev. Respir. Dis. **119**: 785-802.

Roberts, A.M., Bhattacharya, J., Schultz, H.D., Coleridge, H.M. and Coleridge, J.C.G. 1986. Stimulation of pulmonary vagal afferent C fibres by lung edema in dog. Circulation Res. **58**: 512-522.

Rogers, D.F. 1995. Neurokinin receptors subserving airway secretion. Can. J. Physiol. Pharmacol. **73**: 932-939.

Rose, R.D., Koerber, H.R., Sedivec, M.D. and Mendel, M.N. 1986. Somal action potential duration differs in identified primary afferents. Neurosci. Lett. **63**: 259-264.

Rosenfeld, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W., Sawchenko, P.E., Rivier, J., Wale, W.W. and Evans, R.M. 1983. Production of a novel neuropeptide encoded by calcitonin gene via tissue-specific RNA processing. Nature **304**: 129-135.

Ruffin, R.E., Dolovich, M.B., Wolff, R.K. and Newhouse, M.T. 1978. The effects of preferential deposition of histamine in the human airway. Am. Rev. Respir. Dis. **117**: 485-492.

Russel, J.A., and Lai-Fook, S. J. 1979. Reflex bronchoconstriction induced by capsaicin in the dog. J. Appl. Physiol. **47**: 961-967.

Said, S.I. 1982. Vasoactive peptides in the lung, with special reference to vasoactive intestinal peptide. Exp. Lung Res. **3**: 343-348.

Saito, A., Kimura, K. and Goto, K. 1986. Calcitonin gene-related peptide as a potential neurotransmitter in the guinea-pig right atrium. Am. J. Physiol. **250**: H693-H698.

Sanjar, S., Kristersson, A., Mazzoni, L., Morley, J. and Schaeublin, E. 1990. Increased airway reactivity in the guinea pig follows exposure to intravenous isoprenaline. J. Physiol. **425**: 43-54.

Santicioli, P., Maggi, C.A. and Meli, A. 1986. Functional evidence for the existence of capsaicin-sensitive innervation in the rat urinary bladder. J. Pharm. Pharmacol. **38**: 446-451.

Santicioli, P., Maggi, C.A., Geppetti, P., Del Bianco, E., Theodorsson, E. and Meli, A. 1988. Release of calcitonin gene-related peptide-like immunoreactivity from organs of the genitourinary tract in rats. Neurosci. Lett. **92**: 197-201.

Saria, A., Martling, C.R., Dalsgaard, C.J. and Lundberg, J.M. 1985. Evidence for substance P-immunoreactive spinal afferents that mediate bronchoconstriction. Acta. Physiol. Scand. **125**: 407-414.

Saria, A., Martling, C.R., Yan, Z., Theodorsson-Norheim, E., Gamse, R. and Lundberg, J.M. 1988. Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethyl piperazinium, and vagal nerve stimulation. Am. Rev. Respir. Dis. **137**: 1330-1335.

Saria, A., Martling, C.R., Yan, Z., Theodorsson-Norheim, E., Gamse, R., Hua, X.Y. and Lundberg, J.M. 1987. Coexisting peptides in capsaicin-sensitive sensory neurons: release and action in the respiratory tract of the guinea-pig. Acta. Physiologica Hungarica **69**: 421-424.

Saria, A., Lundberg, J. M., Hua, X. and Lembeck, F. 1983. Capsaicin-induced substance P release and sensory control of vascular permeability in the guinea-pig ureter. Neurosci. Lett. **41**: 167-172.

Saria, A., Theodorsson-Norheim, E., Gamse, R. and Lundberg, J.M. 1985. Release of substance P- and substance K-like immunoreactivities from the isolated perfused guinea-pig lung. Eur. J. Pharmacol. **106**: 207-208.

Seifert, H., Chesnut, J., De Souza, E., Rivier, J. and Vale, W. 1985. Binding sites for calcitonin gene-related peptide in distinct areas of rat brain. Brain Res. **346**: 195-198.

Shaw, N.E., Foulkes, R., Andrew, D.P., Brown, D.T. and Hughes, B. 1992. The effects of monoclonal antibodies to calcitonin gene-related peptide (CGRP) on CGRP-induced vasodilation in pig coronary artery rings. Br. J. Pharmacol. **106**: 196-198.

Sheller, J.R., Holtzman, M.J., Skoogh, B. and Nadel, J.A. 1982. Interaction of serotonin with vagal- and Ach-induced bronchoconstriction in canine lungs. J. Appl. Physiol. **52**: 964-966.

Sheng, H., Ishii, K. and Murad, F. 1991. Generation of an endothelium-derived relaxing factor-like substance in bovine tracheal smooth muscle. Am. J. Physiol. **260**: 489-493.

Shigemoto, R., Yokota Y., Tsuchida K. and Nakanishi S. 1990. Cloning and expression of a rat neurokinin K receptor cDNA. J. Biol. Chem. **265**: 623-8.

Shioya, T., Solway, J., Munoz, N.M., Mack, M. and Leff, A.R. 1987. Distribution of airway contractile responses within the major diameter bronchi of the lung during exogenous bronchoconstriction. Am. Rev. Respir. Dis. **135**: 1105-1111.

Shore, S.A. and Drazen, J.M. 1988. Airway responses to SP and SP fragments in the guinea-pigs. Pulmonary Pharmacol. **1**: 113-118.

Shore, S.A. and Drazen, J.M. 1991. Relative bronchoconstrictor activity of neurokinin A and neurokinin A fragments in guinea-pigs. J. Appl. Physiol. **71**: 452-457.

Shore, S.A., Martins, M.A. and Drazen, J.M. 1992. Effect of NEP inhibitor SCH32615 on airway responses to intravenous SP in guinea-pigs. J. Appl. Physiol. **73**: 1847-1853.

Skofitsch, G., Savitt, J.M. and Jacobowitz, D.M. 1985. Suggestive evidence for a functional unit between mast cells and substance P fibers in the rat diaphragm and mesentery. Histochemistry **82**: 5-8.

Small, R.C., Chiu, P., Cooks, S.J., Foster, R.W. and Isaac, L. 1993. Beta-adrenoceptor agonists in bronchial asthma: Role of potassium channel opening in mediating their bronchodilator effects. Clin. Exp. Allergy **23**: 802-818.

Smiths, J.C., Butler, J.P. and Hoppin, F.G. 1984. Contribution of three structures to lung elastic recoil. J. Appl. Physiol. **57**: 1422-1429.

Snider, M.R., Constantine, J.W., Lowe, J.A., Longo, K.P., Label, W.S., Woody, H.A., Drazda, S.E., Desai, M., Vinick, F.J., Spencer, R.B. and Hess, H.J. 1991. A potent nonpeptide antagonist of the substance P (NK1) receptor. Science **251**: 435-439.

Spitzer, W.O., Suissa, S., Ernst, P., Horwitz, R.I., Habbick, B., Cockcroft, D., Boivin, J.F., McNutt, M., Buist, A.S. and Rebuck, A.S. 1992. The use of beta-agonists and the risk of death and near death from asthma. New. Eng. J. Med. **326**: 501-506.

Spriggs, T.L.B. 1965. The effect of anesthesia induced by urethane or phenobarbitone upon the distribution of peripheral catecholamines in the rat. Br. J. Pharmacol. **24**: 752-758.

Steenbergh, P.H., Hoppener, J.W.M., Zandberg, J., Lips, C.J.M. and Jansz, H.S. 1985. A second human calcitonin/CGRP gene. FEBS Lett. **183**: 403-407.

Stephens, N.L., Meyers, J.L. and Cherniack, R.M. 1968. Oxygen, carbon dioxide, H^+, and bronchial length-tension relationships. J. Appl. Physiol. **25**: 376-383.

Stewart, A.G., Thompson, D.C. and Femesy, M.R. 1984. Involvement of capsaicin-sensitive afferent neurons in a vagal-dependent between leukotriene D_4 and histamine on bronchomotor tone. Agents Actions **15**: 500-505.

Stransky, A., Szereda-Przestaszewska, M. and Widdicombe, J.G. 1973. The effects of lung reflexes on laryngeal resistance and motoneurone discharge. J. Physiol. **231**: 417-438.

Stretton, C.D., Belvisi, M.G. and Barnes, P.J. 1989. Neuropeptide Y modulates nonadrenergic noncholinergic neural bronchoconstriction in vivo and in vitro. Br. J. Pharmacol. **98**: 781P.

Stretton, C.D. and Barnes, P.J. 1988. Modulation of cholinergic neurotransmission in guinea-pig trachea by neuropeptide Y. Br. J. Pharmacol. **93**: 672-678.

Struthers, A., Brown, M.J., McDonald, D.W., Beacham, J.L., Morris, H.R. and McIntyre, I. 1986. CGRP: a potent endogenous vasodilator in man. Clin. Sci. **70**: 389-392.

Suresh, M.R., Cuello, A.C. and Milstein, C. 1986. Bispecific monoclonal antibodies from hybrid hybridoma. Meth. Enzymol. **121**: 210-228.

Szallasi, A. and Blumberg, P.M. 1992. Vanilloid receptor loss in rat sensory ganglia associated with long term desensitization to resinferatoxin. Neurosci. Lett. **136**: 51-54.

Szallasi, A. and Blumberg, P.M. 1993. Mechanisms and therapeutic potential of vallinoids (capsaicin-like molecules). Adv. Pharmacol. **24**: 123-155.

Szereda-Przestaszewska, M. and Widdicombe, J.G. 1973. The effect of intravascular injections of veratrine on laryngeal resistance to airflow in eats. Q. J. Exp. Physiol. Cognate Med. Sci. **58**: 379-385.

Szolcsanyi, J., 1983. Tetrodotoxin-resistance non-cholinergic contraction evoked by capsaicinoids and piperine on the guinea-pig trachea. Neurosci. Lett. **42**: 83-87.

Szolcsanyi, J., 1984a. Capsaicin and neurogenic inflammation: history and earlier findings. *In: Antidromic Vasodilation and Neurogenic Inflammation,* (Szolcsanyi, J. and Lembec, F. eds.), pp: 7-26 Academiai Kiado, Budapest.

Szolcsanyi, J. 1984b. Capsaicin-sensitive chemoceptive neural system with dual sensory-effect function. Budapest Academiai Kiado 26-52.

Szolcsanyi, J. and Jancso-Gabor, A. 1975. Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain-producing potency of pungent agents. Arzneimittel-Forschung. **25**: 1877-1881.

Tan, Y.K. and Soldin, S.J. 1987. Analysis of salbutamol enantiomers in human urine by chiral high-performance liquid chromatography and preliminary studies related to stereoselective disposition kinetics in man. J. Chromatography **422**: 187-195.

Taylor, S.M., Pare, P.D. and Schellenberg, R.R. 1984. Cholinergic and nonadrenergic mechanisms in human and guinea-pig airways. J. Appl. Physiol. **56**: 958-965.

Thompson, J.E. and Sheppard, D. 1988. Phosphoramidon potentiates the increase in lung resistance mediated by tachykinin in guinea-pigs. Am. Rev. Respir. Dis. **137**: 337-340.

Tousignant, C., Chan, C.C., Guevremont, D., Brideau, C., Hale, J.J., MacCoss, M. and Rodger, I.W. 1993a. NK2 receptors mediate plasma protein extravasation in guinea-pig lower airways. Br. J. Pharmacol. **108**: 383-386.

Tousignant, C., Chan, C.C., Young, D., Guevremont, D. and Rodger, I.W. 1993b. Neurokinin receptor mediated plasma extravasation in guinea pig and rat airways: comparison of 125I-labelled human fibrinogen and 99mTc-labelled human serum albumin as markers of leakage. Can. J. Physiol. Pharmacol. **71**: 506-511.

Tshikawa, T., Okamura, N., Saito, A., Masaki, T. and Goto, K. 1988. Positive inotropic effect of calcitonin gene-related peptide mediated by cyclic AMP in the guinea-pig heart. Circ. Res. **63**: 726-734.

Tucker, J.F., Brane, S.R., Charalambons, L., Hobbs, A.J. and Gibson, A. 1990. L-N^G-Nitro arginine inhibits non-adrenergic, non-cholinergic relaxation of guinea-pig isolated tracheal smooth muscle. Br. J. Pharmacol. **100**: 663-668.

Turner, A.J., Matsas, R. and Kenny, A.J. 1985. Are there neuropeptide-specific peptidases? Biochem. Pharmacol. **34**: 1347-1356.

Uhl, R.R. and Lewis, F.J. 1974. Digital computer calculation of human pulmonary mechanics using a least square fit technique. Comput. Biomed. Res. **7**: 489-495.

Umeno, E., Hirose, T. and Nishima, S. 1992. Pretreatment with aerosolized capsaicin potentiates histamine-induced bronchoconstriction in guinea-pigs. Am. Rev. Respir. Dis. **146**: 159-162.

Van-Heyningen, V., Brock, D.J.H. and Van-Heyningen, S. 1983. A simple method for ranking the affinities of monoclonal antibodies. J. Immunol. Meth. **62**: 147-153.

Van Euler, U.S. and Gaddum, J.H. 1931. An unidentified depressor substance in certain tissue extracts. J. Physiol. **72**: 74-87.

Von-Essen, S.G., Renard, S.I. and O'Neill D. 1992. Bronchial epithelial cells release neutrophil chemotactic activity in response to tachykinins. Am. J. Physiol. **263**: L226-L231.

Walsh, M.P. 1991. Calcium-dependent mechanisms of regulation of smooth muscle contraction. Biochem. Cell Biol. **69**: 771-800.

Walle, U.K., Pesola, G.R. and Walle, T. 1993. Stereoselective sulphate conjugation of salbutamol in humans: comparison of hepatic, intestinal and platelet activity. Br. J. Clin. Pharmacol. **35**: 413-418.

Wang, Z-L, Bramley, A.M, McNamara, A., Paré, P. and Bai., T. 1994. Chronic fenoterol exposure increases in vivo and in vitro airway responses in guinea pigs. Am. J. Respir. Crit. Care Med. **149**: 960-965.

Wang, J.X., Bray, A.M., Dipasquale, A.J., Mehji, N.J. and Geysen, H.M. 1993a. Systematic study of substance P analogs. I. Evaluation of peptides synthesized by the multipin method for quantitative receptor binding assay. Int. J. Peptide Protein Res. **42**: 384-391.

Wang, J.X., Dipasquale, A.J., Bray, A.M., Mehji, N.J., Spellmeyer, D.C. and Geysen, H.M. 1993b. Systematic study of substance P analogs. II. Rapid screening of 512 substance P stereoisomers for binding to NK1 receptor. Int. J. Peptide Protein Res. **42**: 92-99.

Watson, J.W., Conklyn, M. and Showell, H.J. 1990. IgG_1-mediated acute pulmonary hypersensitivity response in the guinea-pig. Am. Rev. Respir. Dis. **142**: 1093-1098.

Weiner, N. and Taylor. P, 1985. Neurohoumoral transmission: the autonomic and somatic motor nervous system. *In: The pharmacological basis of therapeutics.* (Gilman, A.G., Oodman, L.S., Rall, T.W. and Mura, F., eds.), 7th ed. pp: 66-127, New York: MacMillan.

White, S.R., Sands, M.F. and Murphy, T.M. 1987. Homeostatic regulation of airway smooth muscle tone by catecholamine secretion in swine. J. Appl. Physiol. **62**: 972-977.

Winter, J., Dray, A., Wood, J.N., Yeast, J.C. and Bevan, S. 1990. Cellular mechanism of action of resinferatoxin: a potent sensory excitotoxin. Brain Res. **520**: 131-140.

Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeast, J. and Bevan, S. 1988. Capsaicin-induced ion flux in dorsal root ganglion cells in culture. J. Neurosci. **8**: 3208-3220.

Yiangou, Y., Di Marzo, V., Spokes, R.A., Panico, M., Morris, H.R. and Bloom, S.R. 1987. Isolation, characterization, and pharmacological actions of peptide histidine valine 42, a novel prepro-vasoactive intestinal peptide-derived peptide. J. Biol. Chem. **262**: 14010-14013.

Zaagsma, J., Van Amsterdam, R.G.M., Van Der Heijden, P.G.C.M., Van Der Schaar, M.W.G., Verwey, W.M. and Veenotra, V. 1987. Adrenergic control of airway function. Am. Rev. Respir. Dis. **136**: 545-550.

Zaidi, M., Bevis, P.J., Abeyasekera, G., Girgis, S.I., Wimalawansa, S.J., Morris, H.R. and MacIntyre, I. 1986. The origin of circulating calcitonin gene-related peptide in the rat. J. Endocrinol. **110**: 185-190.

Zaidi, M., Brain, S.D., Tippins, J.R., Chambers, T.J., Morris, H.R. and MacIntyre, I. 1990b. Structure-activity relationship of human calcitonin gene-related peptide. Biochem. J. **269**: 775-778.

Zaidi, M., Moonga, B.S., Bevis, P.J., Bascal, Z.A. and Breimer, L.H. 1990a. The calcitonin gene peptides: biology and clinical relevance. Critical Rev. Clin. Lab. Sci. **28**: 109-174.

Zaidi, M., Chambers, T.J., Gaines Das, R.E., Morris, H.R. and MacIntyre, I. 1987. A direct effect of calcitonin gene-related peptide on isolated osteoclasts. J. Endocrinol. **115**: 511-516.

Zhao, F.Y., Saito, K., Konish, S., Guo, J.Z., Murakoshi, T. Yoshioka, K. and Otsuka, M. 1993. Involvement of NK1 receptors in synaptic transmission in the guinea-pig coeliac ganglion. Neurosci. Res. **18**: 245-248.